Studies of promiscuous activity in *Escherichia coli* adenylosuccinate synthetase

by

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LIST OF ABBREVIATIONS

NAD – nicatinamide adenine dinucleotide

FAD – flavin adenine dinucleotide

CoA – Coenzyme A

AMP, ADP, ATP – adenosine mono-, di-, triphosphate

GMP, GDP, GTP – guanosine mono-, di-, triphosphate

XMP – xanthosine monophosphate

cAMP – cyclic AMP

cGMP – cyclic GMP

PRPP – 5-phospho-α-D-ribosyl-1-pyrophosphate

IMP – inosine monophosphate

6-PIMP – 6-phosphoryl-IMP

SAMP – succinyl-AMP

PFK – phosphofructokinase

OAA – oxaloacetic acid

K_m – Michaelis constant

k_{cat} – catalytic constant

pI – isoelectric point

PNC – purine nucleotide cycle

E. coli – *Escherichia coli*

ppGpp – guanosine 5'-diphosphate-3'-diphosphate

$F16P_2$ – fructose-1,6-bisphosphate

ABSTRACT

The first committed step in *de novo* AMP biosynthesis is catalyzed by adenylosuccinate synthetase (AMPSase). AMPSase utilizes GTP as an energy source to ligate IMP and aspartate in a divalent metal cation-dependent reaction. This reaction proceeds via a 6-phosphoryl-IMP intermediate, which is potentially reactive toward a broad range of substrates. Glycine and other amino acids, as well as hydroxylamine derivatives, participate in AMPSase catalyzed ligations to IMP. The limiting factor governing the viability of a compound as a substrate for AMPSase arises from the intrinsic GTPase activity of the enzyme. Hence, compounds reacting slower than the progress of GTP hydrolysis are not substrates. Studies on this GTPase activity revealed distinct hydrolysis rates in the absence or presence of IMP. Isotope exchange confirmed that 6-PIMP is formed and subsequently hydrolyzed, accounting for the increased GTPase activity seen in the presence of IMP. Other than hydroxide, only amino-nucleophiles have been observed to react. Kinetic studies on the reaction of alternate substrates for aspartate and on inhibition of the aspartate reaction in AMPSase suggest that Ca^{2+} does indeed "open" the active site when bound compared to Mg^{2+}. Furthermore, binding of glycine to the enzyme is unaffected by which divalent cation is present, implying that the α-carboxyl moiety of amino acid substrates other than aspartate do not directly interact with the bound cation. The different K_m values observed for aspartate in the Mg^{2+}- and Ca^{2+}-catalyzed reactions thus stem from interactions with the metal putatively forced by the binding of the β-carboxyl group of aspartate.

CHAPTER 1: GENERAL INTRODUCTION

Life in its most basic terms is a harmony of chemical reactions, made possible and orchestrated by enzymes. Enzymes, however, are not simply wanton catalysts. While almost all enzymes exhibit remarkable specificity for their substrates, many also display the ability to be regulated, whether by feedback/forward effects, allosteric modulation, chemical modification, or protein-protein interactions. In this way, cellular metabolism becomes an elegant, albeit infinitely complex, phenomenon to study. To unravel the mysteries of metabolism, it is thus necessary to focus on individual systems, building knowledge of the whole process one piece at a time.

Purine metabolism is one system that is of particular value to study. Purine nucleotides are absolutely essential to every living organism. They are constituents of DNA, RNA, and major coenzymes such as NAD, FAD and CoA. ATP and GTP are the energy currency of the cell, driving every process from DNA replication to protein synthesis to glycolysis. This energy source is also tapped by the many kinase reactions involved in cellular signal transduction, an area in which cAMP and cGMP also play prominent roles. Thus, not only is the presence of purines critical; delicate regulation maintaining specific purine levels is also necessary for proper function of the cell.

De novo purine biosynthesis can be divided into two parts. The first part begins with activation of ribose-5-phosphate to PRPP, and culminates ten steps later at a metabolic crossroads with the formation of IMP. The second part of the de novo pathway overlaps with the purine salvage pathway, where newly formed IMP faces two possible fates: reaction with IMP dehydrogenase on the way to forming GMP, or reaction with adenylosuccinate

synthetase [IMP:L-aspartate ligase (GDP-forming), EC 6.4.3.3], which catalyzes the first committed step in AMP biosynthesis (1). Fig. 1 shows an overview of purine metabolism.

Underscoring the importance of adenylosuccinate synthetase is the fact that it is present in almost every known organism and tissue, except some intracellular parasitic bacteria and the mature erythrocyte (2, 3). Even organisms which lack a de novo purine synthesis pathway, such as leischmanial and trypanosomal parasites, depend upon the role of adenylosuccinate synthetase in purine salvage pathways (4, 5). There is significant sequence conservation across all species, ranging from approximately 40% between eubacterial and mammalian synthetases to greater than 95% between like isozymes of human and mouse (2, 6-10).

Mammals possess two different forms of adenylosuccinate synthetase, which are referred to as the acidic (also liver, or AdSS2) and basic (also muscle, or AdSS1) isozymes, following from their different pIs (5.9 and 8.9 respectively). Additionally, these isozymes differ in kinetic parameters, regulation, and tissue distribution (1, 2, 9-14). Recent cloning and over-expression of both the acidic and basic mouse isozymes allowed the detailed characterization of differences between the two, and confirmed the general assignment of separate roles for the two isozymes (1, 7, 9, 15).

Based on tissue distribution and on early studies of each enzyme, it was hypothesized that the acidic isozyme functions in a de novo capacity, while the basic enzyme is involved in salvage pathways (1). The acidic enzyme resembles the *Escherichia coli* synthetase more closely in sequence and behavior, having a higher affinity for IMP and being potently inhibited by AMP. The basic enzyme is largely indifferent to AMP levels, but has a higher K_m for IMP, lower K_m for aspartate, and displays substrate inhibition by IMP. Both are

inhibited by fructose-1,6-bisphosphate (1, 9). These behaviors are consistent with the ostensible purposes of each synthetase, and the basic enzyme especially seems to be fine-tuned for one salvage pathway in particular, known as the PNC.

The PNC, or purine nucleotide cycle, is found only in vertebrates. It has been shown to occur in muscle, liver, brain, pancreatic islets, and kidney tissues (16-21). The process involves the typical progression of IMP to AMP via action of adenylosuccinate synthetase and adenylosuccinate lyase, with IMP subsequently being re-formed by AMP deaminase. The net reaction for this cycle is: L-aspartate + GTP + H_2O → Fumarate + GDP + P_i + NH_3 (16).

Several rationales have been put forth to explain the cell's utilization of the PNC: 1) to convert AMP to IMP so as to "pull" the adenylate kinase reaction forward, aiding in replenishment of depleted ATP stores (as in exercising muscle), 2) to liberate ammonia from amino acids via aspartate (particularly relevant in kidney), 3) to regulate glycolysis by adjusting levels of IMP, AMP, and NH_3, of which the former activates glycogen phosphorylase, and the latter two inhibit PFK, and 4) to compensate for loss of anaplerotic activity in cells lacking pyruvate carboxylase by providing fumarate to the Krebs cycle rather than oxaloacetic acid (1, 11, 16, 23).

It has been shown that in liver tissue, the typical ratio of acidic to basic forms of adenylosuccinate synthetase is 60:40. During regeneration after injury, that ratio increases to 80:20, arguing for the role of the acidic enzyme in the *de novo* pathway (21, 22). Bolstering the case for the basic enzyme's involvement in the PNC is the fact that it is the only form found in skeletal and cardiac muscle, and has been shown not only to be important in alleviating muscle fatigue (presumably through the PNC), but also to bind directly to

contractile muscle proteins (23-25). Yet, the acidic isozyme predominates in tissues where the PNC has been shown to be active, such as brain and kidney, and de novo AMP biosynthesis occurs in muscle. Thus, while both mammalian isozymes seem tailored to separate roles in cellular purine metabolism, it is nevertheless unwarranted to define those roles as mutually exclusive.

In physiologic terms, the relative catalytic ability of adenylosuccinate synthetase may be important. The *E. coli* enzyme has a measured k_{cat} of ~1.4 s^{-1}, one of the lowest recorded in the literature for an enzyme. From estimates of the total adenine pool and total synthetase present in the cell, the minimum calculated doubling time for *E. coli* based on adenylosuccinate synthetase activity would be 21 minutes. This value is very close to observed doubling times for *E. coli* under optimal conditions (2).

Kinetic and chemical mechanism of adenylosuccinate synthetase

The kinetic mechanism of adenylosuccinate synthetase is essentially sequential rapid-equilibrium random ter-ter (26). However, evidence also exists suggesting there is a strongly preferred binding order, whereby IMP and GTP add randomly, followed by the addition of aspartate (27). Recently though, studies done on the *Plasmodium falciparum* synthetase have shown that its mechanism is ordered, with initial binding of IMP being followed by GTP and then aspartate. This sets it apart from all other adenylosuccinate synthetases studied thus far (28).

Three different mechanisms have been proposed for the reaction, all of which account for a random model (29-31). Even so, overwhelming experimental evidence points to the mechanism proposed by Lieberman, which involves a 6-PIMP intermediate (27, 32-34). The

first step entails transfer of the γ-phosphoryl of GTP to IMP. This occurs when the N1 hydrogen of IMP is extracted, leading to formation of an activated 6-oxyanion for nucleophilic attack of GTP, after which GDP becomes the leaving group. In the next step, another nucleophilic attack occurs as the free electron pair of the unprotonated amino group of aspartate attacks the C6 position of 6-PIMP, displacing the 6-phosphoryl group. The local pH environment is thus of great significance, as is enzymatic ability to maintain a neutral amino species on the substrate, since aspartate would be entirely unreactive if its amino group is protonated. Fig. 6 in Chapter 2 shows the reaction mechanism of AMPSase.

The existence of 6-PIMP has been confirmed both by crystallography and by isotope exchange experiments. 6-PIMP and 6-thio-PIMP have been trapped in the active site of the synthetase, and a β-γ phosphate bridging ^{18}O in GTP exchanges only in the presence of IMP. (32-34, 68 *et al*). Formation of an activated intermediate such as 6-PIMP has substantial consequences for specificity and catalysis. Any available nucleophile could in theory react with the intermediate, requiring the enzyme to not only bind substrates in the aspartate site with high specificity, but also to have the ability to exclude any other potential nucleophile from contact with active site 6-PIMP.

Specificity and inhibition of adenylosuccinate synthetase

Adenylosuccinate synthetase is a peripheral member of the G-protein super-family. This classification stems from the conserved guanine and phosphate binding regions it contains. The guanine recognition moiety, or (N/T)KXD box (69), is critical in recognition of GTP, as is evident when an Asp333 to asparagine mutation in *E. coli* changes specificity of the enzyme to XTP. From a functional standpoint, specificity for GTP over other nucleotide

triphosphates is not important, so long as there is efficient transfer of the γ-phosphoryl to IMP. In fact, the XTP mutant has higher activity than the wild-type GTP-specific enzyme (49). Physiologically though, as adenylosuccinate synthetase plays a key role in maintaining adenine/guanine ratios, GTP specificity is important to proper function in the context of cellular metabolism. In addition to GTP, GTPγS and 2'deoxyGTP have been shown to serve as substrates (12, 50). The synthetase also contains variations of the phosphate binding consensus sequence GXXXXGK, known as the "P-Loop." This loop is critical to the function of the "on/off" switch of G-proteins, as hydrolysis of bound GTP to bound GDP inactivates the G-protein (36, 70). Indeed, recent studies have shown that *E. coli* adenylosuccinate synthetase possesses an intrinsic GTPase activity (pending publication).

The GTP site is feedback inhibited by both GMP and GDP with K_is similar to the K_m for GTP (~20μM). 2'-deoxyGMP/GDP and β-D-arabinosyl-GMP bind much more poorly to the enzyme, but can exert the same inhibitory influence as their ribose counterparts at high concentrations (1, 13, 15, 37). NO_3^- inhibits synergistically with GDP, mimicking the γ-phosphoryl of GTP (32). The formation of guanosine 5'-diphosphate-3'-diphosphate (ppGpp) during stringent response in *E. coli* greatly reduces synthetase activity, and represents an example of deliberate regulation by a compound other than a direct metabolite of the enzyme (51, 52). Interestingly, inhibition by GDP or by GMP of the *P. falciparum* synthetase is uncompetitive with respect to IMP. This is due to the synthetase having an ordered mechanism, which is unique among all adenylosuccinate synthetases studied as of yet. Even more striking is the fact that $F16P_2$ (inhibitory for every other synthetase) is an activator when accompanied with conditions of limiting GTP (29).

Specificity for IMP is narrow, and most compounds are inhibitory, rather than reactive, when binding the IMP site. 2'-deoxyIMP and β-arabinosyl-IMP have similar K_ms to IMP (5-54μM), but much lower rates of catalysis. This may partly be due to the fact that there is a subsequent increase in the K_m for aspartate when these substrates bind, and assays simply were not done at saturating concentrations of aspartate. When aspartate conditions are adjusted accordingly, full activity is seen with 2'-deoxyIMP (38, 9). Adenylosuccinate synthetases from *Leishmania donovani* and *Trypanosoma cruzi* can turn over two compounds (8-aza-IMP and allopurinol ribonucleotide) that are weakly inhibitory to the mammalian forms. The product resulting from catalysis with the allopurinol compound is incorporated eventually into the organisms' RNA, and becomes fatal (39-40). For this reason allopurinol ribonucleotide is a good anti-leishmanial and anti-trypanosomal agent.

The most physiologically relevant inhibition with respect to IMP comes from AMP and SAMP, which exert their feedback through this site. AMP has K_is ranging from 47-170μM, while SAMP has K_is close to K_ms for IMP. The basic isozyme is less sensitive to AMP, but is regulated by IMP itself, through substrate inhibition (9, 15, 36). XMP also inhibits with respect to IMP, with a K_i similar to that of AMP. 6-mercaptopurine ribonucleotide, an important nucleotide metabolism inhibitor, has been shown to competitively inhibit the enzyme with respect to IMP (36, 37).

One of the most potent inhibitors (K_i = 0.2μM) of adenylosuccinate synthetase is a compound called L-alanosyl-5-amino-4-imidazolecarboxylic acid (alanosyl-AICOR), which results from the incorporation of L-alanosine instead of aspartate in the reaction catalyzed by SAICAR synthetase (41). Still, this compound is not as potent as another inhibitor (K_i = 0.1μM) that binds the IMP site, called hydantocidin 5'-phosphate, which is a naturally

occurring compound with herbicidal properties secreted by *Streptomyces hydroscopicus* (43, 44).

Much broader specificity exists in adenylosuccinate synthetase for aspartate than for the other two main substrates. Given the reactivity of 6-PIMP, and the relative simplicity of aspartate itself, this is not surprising. The enzyme has much lower affinity (Km ~ 200μM) for aspartate than for its other two substrates (1). Previously confirmed alternate substrates for aspartate are hydroxylamine, L-alanosine, alanine-3-nitronate, DL-threo-β-fluoroaspartate, and β-cysteine sulfinate (45-47). Alanosine has antibiotic, antitumor, and immunosuppressive effects, and while the effect of alanosine as a substrate of SAICAR synthetase (production of alanosyl-AICOR) can be quite powerful, it is relatively benign with respect to adenylosuccinate synthetase (46). The product resulting from catalysis with β-cysteine sulfinate has been shown to be involved in heavy metal tolerance in the fission yeast *Schizosaccharomyces pombe*. This SAMP analog cannot be cleaved by adenylosuccinate lyase, but instead is believed to be an intermediate or carrier in a transfer pathway of sulfides destined for incorporation into phytochelatin complexes which bind heavy metals (48).

Recently, the extent of adenylosuccinate synthetase's catalytic range has been exposed further, with demonstration that under rigorous conditions, the ligation reaction can be catalyzed with glycine, alanine, serine, glutamate, methoxylamine, ethoxylamine, and carboxymethoxylamine (pending publication). As evidence continues to surface demonstrating the breadth of reactions the enzyme can catalyze in substitution for aspartate, and the potential versatility of resulting SAMP analogs, the study of adenylosuccinate synthetase becomes an increasingly compelling.

Yet, while the greatest catalytic promiscuity of the enzyme involves substrates in place of aspartate, one of the most potent inhibitors known to date binds in the aspartate pocket of the active site. Hadacidin (N-formyl-N-hydroxyaminoacetic acid) is a natural antibiotic isolated from the fermentation product of *Penicillin frequentans*, and has K_is ranging from 0.3-6.3μM (31, 37, 53). Other inhibitors with respect to aspartate tend to contain the dicarboxylic acid moiety of the natural substrate. Succinate, oxaloacetate, malonate, and phosphoenol pyruvate have K_is of 1.8mM, 0.5mM, 0.9mM, and 0.2mM respectively in the synthetase from *Azotobacter Vinelandii* (31). Studies on the *E. coli* enzyme have shown some of these to be less effective, however. Judging from two unsaturated dicarboxylic acids tested, maleate (*cis*-butenedioic acid) is inhibitory (K_i = 3.1mM), while fumarate (trans-butenedioic acid) is not, indicating that aspartate probably binds in the *cis* conformation (44).

Fructose-1,6-bisphosphate is a potent inhibitor of adenylosuccinate synthetase and is noncompetitive with respect to all substrates. While most reported K_is are too high to be physiologically relevant, the basic enzyme in mammalian muscle may encounter inhibitory levels of $F16P_2$ in vivo (1, 2, 54). While mouse basic and acidic enzymes are equally inhibited by $F16P_2$ with respect to and in the absence of IMP (K_is ~17μM), the former is much more potently inhibited with respect to GTP and aspartate (K_is 6 μM and 81μM vs. 400μM and 670μM, respectively). There have been two alternate binding sites proposed, one being the IMP pocket in the absence of IMP (mimicking 6-PIMP), and the other being an as yet unidentified allosteric site (9). Of particular note, and mentioned earlier, is the fact that the *P. falciparum* synthetase is activated by high concentrations of $F16P_2$ in the presence of

limiting GTP. This effect is only seen if aspartate is used to initiate the reaction, presumably because aspartate binds the allosteric site otherwise (29).

Adenylosuccinate synthetase requires a divalent metal cation for catalysis. Mg^{2+} is the best activator, followed by Ca^{2+} and Mn^{2+}, and in some cases Co^{2+}, Ba^{2+} and Cu^{2+} (12, 13, 29, 37, 55). Zn^{2+} has shown conflicting abilities either as an inactivator, or not, but Hg^{2+} universally inactivates the enzyme at low concentrations, presumably reacting with an active site thiol (12, 40, 56, 57). In *E. coli*, it has been shown that different divalent cations can have a marked effect on the kinetic parameters of the enzyme. Generally, the K_ms for substrates are lower in the Ca^{2+} catalyzed reaction, although the k_{cat} is greatly reduced as well. Evidence that there is a second Mg^{2+} necessary for catalysis has also been shown (57). Recent experiments have shown that for some alternate substrates for aspartate, the effects on the kinetic parameters are quite different, at times even reversed (pending publication).

Structure and function of adenylosuccinate synthetase

The active association state of adenylosuccinate synthetase is as a dimer. In solution, the enzyme is in a monomer-dimer equilibrium (K_d = 10μM) that is greatly shifted in favor of dimer in the presence of substrates. One key residue involved in dimerization, Arginine143, has a side chain that projects from one subunit into the active site of the two-fold symmetry-related subunit, and interacts with bound IMP. This not only serves to orient the interface of the two monomers, but also confers sensitivity to substrate binding on the dimerization of the enzyme. Indeed, IMP is the most effective substrate contributing to stabilization of the dimer. Subunit complementation studies using specific mutants such as Arg143 confirmed the rapid

monomer-dimer exchange of adenylosuccinate synthetase in solution, and demonstrated that both active sites can support catalysis (58-61).

Crystals of synthetases from *E. coli* (60-65, 67, 68 et al), *Arabidopsis thaliana* and *Triticum aestivum* (66), mouse muscle (34), and *Plasmodium falciparum* (8) have been described in the literature. In addition to associating as dimers, all synthetases crystallize as dimers and exhibit a common core fold consisting of a 10-stranded β-sheet, with bordering α-helices and loops (2, 60-65). The *E. coli* synthetase has been the most rigorously studied from a crystallographic standpoint, and all further discussion of structure will focus primarily on this enzyme. Fig. 2 is a ribbon model of AMPSase in its fully ligated state.

The binding of IMP alone can organize the active site of *E. coli* adenylosuccinate synthetase. This organization is the result of a cascade of conformational changes centered on the 5'-phosphate of IMP. The IMP Loop (residues 120-130) shifts so that a conserved threonine (Thr129) can interact with the 5'-phosphate. This in turn promotes a well-defined conformation of loop residues 126-129, which then bind to the sidechain of Glu118 at the N-terminus of the IMP Loop, stabilizing the fully ordered loop. Asn38 also interacts with the 5'-phosphate of IMP and in doing so promotes the leverage of the Switch Loop (residues 38-53) into the activated position. Once this movement has occurred, several interactions (GTP Loop:Switch Loop, Asp21:Arg419, and His41:Glu221) further steady the active conformation. This conformation provides a ready binding site for GTP-Mg^{2+}. Finally, Arg143 interacts with the 5'-phosphate as part of the dimerization process discussed earlier. Only the Aspartate Loop remains somewhat disordered when IMP is bound, but in crystal complexes of IMP and hadacidin, the enzyme is in the same conformation as the fully ligated one (61, 62, 67).

GTP binding is mediated by interactions with four regions of the enzyme: 1) the (N/T)KXD box (residues 330-333) , which recognizes the guanine base, 2) a variation of the consensus GXXXXGK sequence, or "P-Loop," which binds the phosphate moieties through backbone amide interactions, 3) the GTP Loop (residues 417-421), which packs against the base, and 4) the Switch Loop, which interacts with the phosphates of GTP/GDP complexed with Mg^{2+} (or other divalent cations, presumably). (2, 60-65, 69, 70).

The guanine recognition box and the P-Loop do not undergo conformational changes upon ligand binding, whereas the GTP Loop undergoes a rigid body shift from its unligated position toward the Switch Loop. The Switch Loop is supported in its inactive conformation by His41 interacting with Asp 21. However, when the GTP Loop engages, Arg419 binds Asp21, releasing His41, which then forms bridging interactions with the γ and β-phosphoryls of GTP. The conformation of the fully ligated active site seems energetically geared toward promotion of the transition state, rather than favorable binding of GTP. Crystals of the *E. coli* synthetase have not grown in the presence of only GDP-Mg^{2+} or GTP-Mg^{2+}, and this may be due to the enzyme avoiding ordering of the active site with only GTP, which would encourage unproductive hydrolysis. (2, 60-65, 69, 70).

Understanding of binding interactions between the Aspartate Loop (residues 298-304) and its true substrate are only tentative, since no structures exist with aspartate in the active site. Hadacidin is used almost exclusively to ligate the aspartate site in crystals, and when present yields an ordered Aspartate Loop. The loop is otherwise disordered. The N-formyl group of hadacidin coordinates with Mg^{2+}, the N-hydroxy group hydrogen bonds with Arg305, and the β-carboxyl group binds with Arg303. While the latter interaction is in all likelihood directly comparable to binding of the β-carboxyl of aspartate, the α-carboxyl of

aspartate was modeled with one oxygen coordinating Mg^{2+}, and the other hydrogen bonding with Arg305. The amino group of aspartate was modeled hydrogen bonding with backbone carbonyl 38 and with its own β-carboxyl group, activating it for nucleophilic attack (61-64). However, the mouse muscle synthetase crystallized with SAMP does not support the activating role of the β-carboxyl group interaction, and the same is likely true for the *E. Coli* enzyme (73). Recent evidence from kinetics with alternate aspartate substrates suggests that aspartate may not interact with the active site Mg^{2+} (or Ca^{2+}), but only with Arg305. Indeed, crystals with only IMP and hadacidin bound displayed ordered Aspartate Loops with binding characteristics identical to fully ligated loops, even in the absence of Mg^{2+} (44, 67).

Mutations of Arg303, 304, and 305 to leucine dramatically increase the K_m for aspartate, as do Thr301 and Val273 mutants. Changing either Arg303 or Arg305 directly interrupts binding to the carboxyl groups of aspartate, while a mutation in Arg304 likely alters the conformation of the backbone, and causes similar disruption through Arg303 and Arg305. Val273 is likely involved in ensuring productive binding of aspartate while also deterring inhibitory binding of other dicarboxylic acids. The Thr301 to alanine mutation results in a dead enzyme by putative loss of hydrogen bonding to the β-carboxyl of aspartate. Assays with hydroxylamine, which does not require hydrogen bonding, resulted in similar k_{cats} for both the wild-type and Thr301A mutant enzymes (44, 71).

The mechanistic basis of catalysis has also been greatly elucidated by crystallographic studies. Structures corresponding to both the initial transition state and the 6-PIMP intermediate steps point to essential roles of several residues. The enzyme ligated with IMP, GDP, hadacidin, Mg^{2+}, and NO_3^- (mimicking the γ-phosphoryl in mid-transfer) served as a model for the first transition state when IMP nucleophilically attacks GTP (62). In this

model, Asp13 serves as the catalytic base, extracting the N1 hydrogen from IMP. The resulting 6-oxyanion is stabilized by Gln224, and during subsequent attack on the γ-phosphoryl of GTP, His41 stabilizes the developing charge on the β-phosphoryl of GTP. Full separation of GDP occurs as His41 (the catalytic acid) relinquishes its hydrogen (68).

Crystal structures with 6-PIMP (or 6-thio-PIMP), GDP, hadacidin and Mg^{2+} serve to illustrate the enzyme in preparation for the second nucleophilic attack by aspartate (66, 69). His41 reorients to interact with the 6-phosphoryl group of 6-PIMP, and presumably serves again as a catalytic acid to assist in phosphate release. Asp13 shifts into the Mg^{2+} coordination sphere, in the process protonating (as another catalytic acid) the N1 position of 6-PIMP (activating the C6 position for nucleophilic attack) (69). The α-amino group of aspartate must be neutral (or possibly anionic), to participate in the attack. The enzyme presumably encourages this through the forced interaction of the α-carboxyl with the active site Mg^{2+}, thereby disrupting zwitterionic stabilization of the charged amino species. It was previously postulated that proton abstraction from the charged amino species of aspartate was carried out by its own β-carboxyl group, but later SAMP-bound crystal structures negated this possibility. (2, 63, 74).

The importance of these residues for catalysis in adenylosuccinate synthetase is evident both from their universal conservation across species, and from kinetic studies with appropriate mutants. All mutants for Asp13, His41, and Gln224 show very little activity or are dead altogether. This, along with the crystallographic data, is strong evidence for the catalytic mechanism proposed (69, 72).

Thesis organization

This thesis is organized into three chapters. The first is a general introduction and literature review of the topic. The second is a manuscript that is to be submitted for publication in a scientific journal. The final chapter focuses on general conclusions and the relevance of the research to both the existing body of work on the enzyme and to the biological sciences overall.

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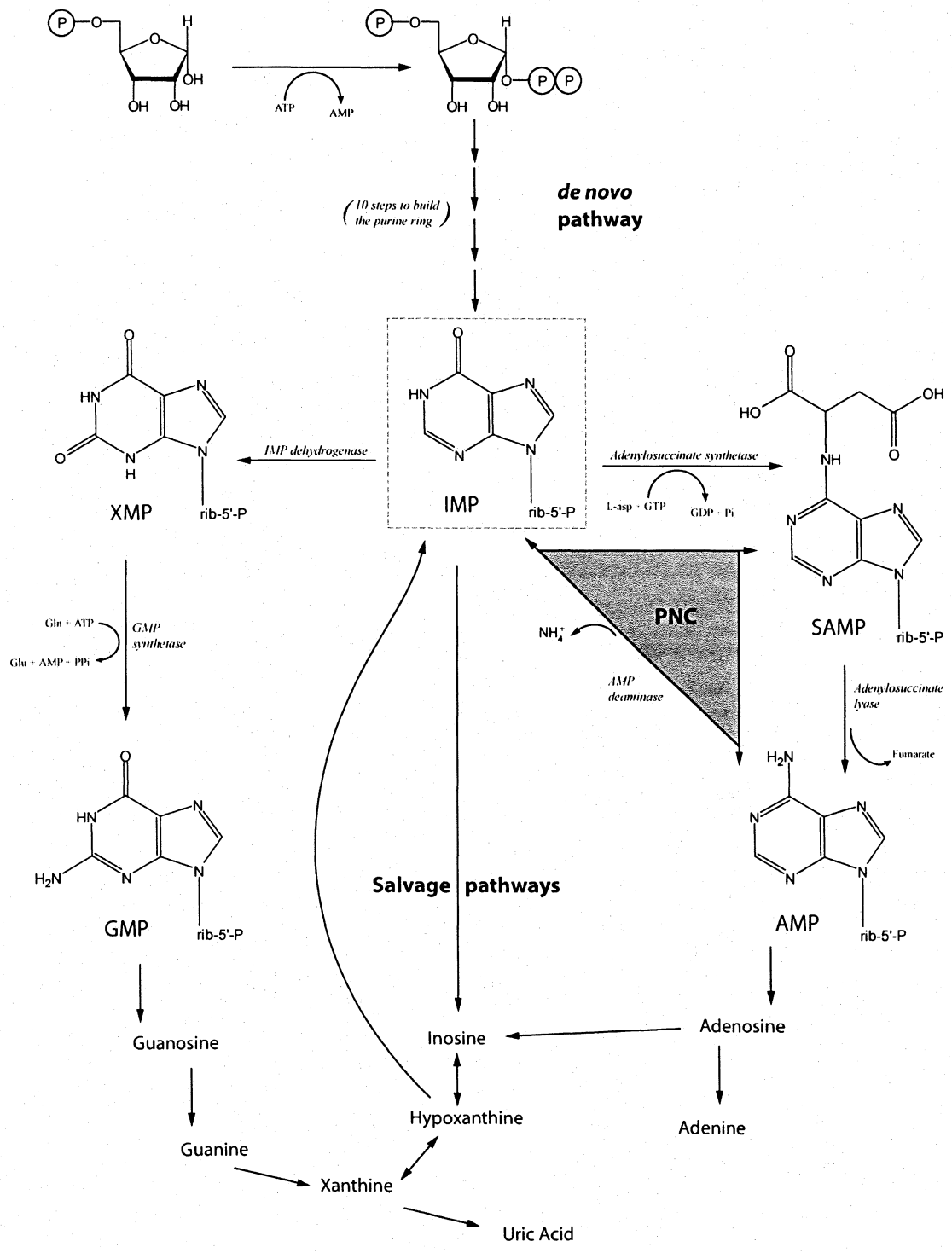

Fig. 1. Purine Metabolism. IMP represents a crossroads of the de novo synthesis and salvage pathways. Adenylosuccinate synthetase catalyzes the first committed step in AMP biosynthesis, and also participates in the purine nucleotide cycle, or PNC.

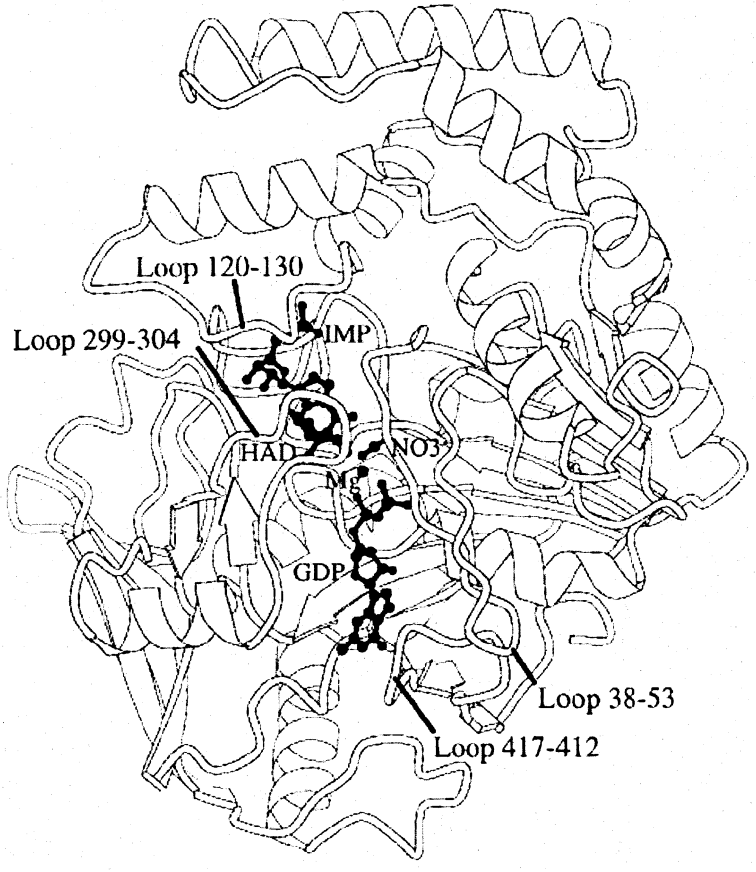

Fig. 2. AMPSase in its fully ligated conformation. The ligands, IMP, GDP, Mg^{2+} (labeled Mg), nitrate (labeled NO3), and hadacidin (labeled HAD) are represented by ball-and-stick structures. Dynamic loops discussed in the text are labeled. Only one monomer of the dimer is shown. This illustration was prepared by MOLSCRIPT (74).

CHAPTER 2: ADENYLOSUCCINATE SYNTHETASE IN THE SYNTHESIS OF N-6-SUBSTITUTED ADENINE NUCLEOTIDES[1]

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Abstract

Adenylosuccinate synthetase catalyzes the first committed step in *de novo* AMP biosynthesis, hydrolyzing GTP in the formation of adenylosuccinate from IMP and L-aspartate. The enzyme putatively stabilizes a positive charge at atom C-6 of a 6-phosphoryl-IMP intermediate, but does little to enhance the nucleophilic properties of the α-amino group of L-aspartate. If so, a variety of nucleophiles should react with enzyme bound intermediate, and yet only hydroxylamine and a few analogs of L-aspartate have been reported as substrates. The present investigation expands the list of substrates of the synthetase: methoxylamine, ethoxylamine, carboxymethoxylamine, glycine, alanine, serine, and glutamate all form N-6 adducts of AMP. The α-carboxyl group of amino acid substrates is a critical determinant, as alcohol analogs of glycine, alanine and serine are not substrates at pH 7.7. Unlike L-aspartate, which exhibits different kinetic parameters for the Mg^{2+}- and Ca^{2+}-activated synthetases, glycine is insensitive to the type of divalent cation activator. Yet glycine exhibits saturation kinetics consistent with the formation of a Michaelis complex. All substrates of the synthetase compete with the hydroxyl anion. The latter causes the net hydrolysis of GTP by IMP-independent and IMP-dependent pathways. The observation of

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catalytic activity with derivatives of hydroxylamine and a variety of α-amino acids is consistent with the proposed chemical mechanism for the synthetase, and suggests an alternative route to the synthesis of N-6-substituted adenines.

Introduction

Adenylosuccinate synthetase (EC 6.3.4.4) catalyzes the first committed step in *de novo* AMP biosynthesis:

$$\text{IMP} + \text{L-aspartate} + \text{GTP} \xleftrightarrow{Mg^{2+}} \text{adenylosuccinate} + \text{GDP} + P_i$$

The enzyme participates in purine nucleotide salvage pathways in parasites responsible for significant world health problems, such as malaria, and in vertebrates is one of three enzymes involved in the purine nucleotide cycle (1-3). Several natural compounds are effective and specific inhibitors of the synthetase. Hadacidin (*N*-formyl-*N*-hydroxyglycine) is an L-aspartate analog that inhibits only adenylosuccinate synthetase (4), and hydantocidin is a natural herbicide that specifically targets the synthetase of plants (5, 6). The synthetase may play a key role in possible treatments for HIV (7), cancer (8), and malaria (9, 10).

The enzyme-catalyzed reaction proceeds by way of an intermediate 6-phosphoryl-IMP (6-PIMP[1]), formed by the transfer of the γ-phosphoryl group of GTP to the 6-O atom of IMP (11, 12). Rate enhancement for this first step likely comes in part through the abstraction of a proton from the N-1 atom of IMP by an aspartyl side chain, which generates the 6-oxyanion of IMP for nucleophilic attack on GTP. Following formation of the intermediate, the ligation occurs as the α-amino group of L-aspartate displaces the phosphoryl group from the C-6 position of 6-PIMP. Rate enhancement for this second step putatively

stems from the generation of the 6-C cation of 6-PIMP, brought about by re-protonation of the N-1 position by the catalytic aspartyl side chain involved in the first step of the reaction (13-16). This activated 6-PIMP intermediate should react with a broad range of nucleophiles, and yet most of the reported participants in the synthetase ligase reaction closely resemble L-aspartate: L-alanosine, alanine-3-nitronate, DL-threo-β-fluoroaspartate, and β-cysteine sulfinate (17-19). The only known substrate of the synthetase differing substantially from L-aspartate is hydroxylamine (32). The existence of an activated 6-PIMP intermediate would be supported by a broadened range of substrates that result in the formation of adenylosuccinate (SAMP) analogs.

A second aspect of the investigation is how the synthetase overwhelmingly selects L-aspartate in preference to other amino acids. Structural models for the complex of 6-PIMP, GDP and L-aspartate based on crystallographic structures of the 6-PIMP•GDP•hadacidin complex (13) and the SAMP•GDP•sulfate complex (29), indicate a crowded environment for the Mg^{2+}-coordinated α-carboxyl group of L-aspartate. When Ca^{2+} replaces Mg^{2+} in assays, the K_m for L-aspartate falls five-fold (31), consistent with the relief of steric crowding due to a larger ionic radius for Ca^{2+} relative to Mg^{2+}. Hence, L-aspartate is a preferred substrate partly because this forced coordination of the catalytic metal effectively destabilizes the zwitterionic state of the amino acid, thus causing the de-protonation of the α-amino group. Amino acids other than L-aspartate may participate in enzyme-catalyzed reactions, but not bind to any catalytic metal (Mg^{2+} or Ca^{2+}), or bind only to the Ca^{2+}-activated enzyme. Hence, substrate specificity of the synthetase could vary with the type of catalytic metal.

This study demonstrates a much broader range of substrates for the synthetase than had been documented previously. The hydroxyl anion, methoxylamine, ethoxylamine,

carboxymethoxylamine, glycine, alanine, serine, and glutamate all form adducts of IMP. Reactions with the hydroxyl anion result in the net hydrolysis of GTP, but occur by distinct pathways, dependent and independent of IMP. Hydrolysis of GTP ultimately defines a limiting rate of reaction, as all potential substrates of the synthetase must compete with the hydroxyl anion for enzyme-bound 6-PIMP. The synthetase may provide a viable alternative to traditional organic synthetic routes for the synthesis of N-6-substituted adenine nucleotides.

Experimental

Materials- Vent polymerase, restriction enzymes, and DNA ligase were from New England Biolabs. Plasmid pET28b, *E. coli* strain BL21(DE3), and nickel-nitrilotriacetic acid agarose were from Novagen Inc. All other reagents were from Sigma unless otherwise noted.

Construction of His-tagged synthetase- A fragment of 1371 base pairs was amplified using the following primers: forward, 5'-CTACGTTACATATG GGTAACAACGTCGTCGTACTG-3' (*Nde* I restriction site underlined) and reverse, 5'-CCGCTCGAGTTACGCGTCGAACGGGTCGCGCAG-3' (*Xho* I restriction site underlined). Insertion of the amplified fragment into corresponding sites of the pET28b expression vector resulted in the plasmid pAdSSEcoli; the construct was checked by sequencing at Iowa State University DNA sequencing facility.

Expression and Purification of Recombinant Enzyme- AMPSase was expressed at 37 °C in *E. coli* BL21 (DE3) cells grown in LB media containing 30 μg/mL Kanamycin. At an OD_{600} of 1.0-1.4 the protein overproduction was induced by the addition of 0.5 mM isopropyl thio-β-D-galactoside (final concentration). Collection of cells was done by centrifugation at 4000xg

for ten minutes at 4 °C, after which the pellet was resuspended in lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, and 10 mM imidazole, pH 8). After lysis by French press, and centrifugation at 24,000xg for 30 min. at 4 °C, sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (36) of samples from supernatant revealed the production of the *E.coli* AdSS protein (~48 kDa), which accounted for approximately 30-40 % of the total soluble protein. The supernatant was loaded onto a nickel-nitrilotriacetic acid-agarose column pre-equilibrated with lysis buffer. Two subsequent ten volume washes were done using buffers differing from the lysis buffer only in the amount of imidazole present (20 mM and then 30 mM). Bound protein was eluted with 300 mM imidazole, and was dialyzed against a buffer containing 50 mM HEPES, 50 mM NaCl, 1 mM dithiothreitol, and 0.5 mM EDTA, pH 7.5. The protein was > 95 % pure as judged by running 20 μg of recombinant AdSS on 10 % polyacrylamide gels (SDS-PAGE) (36).

Enzyme Assay- Protein concentration was determined by the Bradford method (37), using bovine serum albumin as a standard. Enzyme activity for aspartate and alternate substrates was determined by measuring the formation of product by change in OD at 280 nm. All reactions were initiated by addition of enzyme. Assays of the aspartate reaction, and inhibition of the aspartate reaction, were done at 22 °C. Assays contained 300 μM GTP, 500 μM IMP, 5 mM Mg^{2+} or Ca^{2+}, and 40 mM HEPES pH 7.7 held constant. Aspartate concentrations varied from 37.5 μM-1200 μM and 6.25 μM-200 μM for Mg^{2+} and Ca^{2+} respectively. Acetate inhibitor ranged from 75-1200 mM. Enzyme concentrations ranged from 5-30 μg/mL. Assays of the glycine reaction were done at 37 °C. The constant conditions were the same as above, with concentrations of glycine ranging from 6.25-1200 mM, and enzyme concentrations of 515 μg/mL.

Initial Reaction Screening and GTP Hydrolysis Assay- Screening of alternate substrates for aspartate was done by HPLC. Reaction mixtures contained 1 mM IMP, 1 mM GTP, 5 mM Mg^{2+} or Ca^{2+}, 40 mM HEPES pH 7.5, 100 mM alternate substrate, and 2 mg/mL AMPSase. Reactions were incubated at 37 °C for 18 hours and loaded onto a Waters analytical C-18 reverse-phase HPLC column. A standard protocol for separation of 5'-ribonucleotides was employed, running a buffer containing 100 mM KH_2PO_4 and 4 mM t-butyl ammonium hydrogen sulfate, and eluting with a gradient of 0-30% methanol (40). Progression of GTP hydrolysis was also monitored by HPLC. Reaction mixtures consisted of 1 mM GTP, 5 mM Mg^{2+} or Ca^{2+}, 40 mM HEPES pH 7.5, and 2 mg/mL AMPSase, in the absence of, or with 1 mM IMP. Aliquots were taken every 60 minutes for 6 hours. Buffer running conditions were as described above.

Purification and Quantification of AMP analogs- Purification of products from the successful alternate substrate reactions was done by ion-exchange HPLC. Reaction mixtures contained 5 mM IMP, 5 mM GTP, 5 mM Mg^{2+} or Ca^{2+}, 40 mM HEPES pH 7.5, and 2 mg/mL AMPSase. After 24 hr incubation, samples were loaded onto a column containing Super-Q-5PW DEAE anion-exchange resin (TosoHaas) equilibrated with triethylammonium bicarbonate (TEAB). A gradient of 10 mM to 1M TEAB was used to purify AMP analogs from the reaction mix. Collected fractions were lyophilized and redissolved in DI water. Concentration of samples was determined using the ammonium molybdate assay to measure the amount of inorganic phosphate released upon digestion with alkaline phosphatase (39). UV spectrum scans in 40 mM HEPES pH 7.5 revealed the λ_{max} and allowed calculation of extinction coefficients.

Mass Spectrometry Analysis- MS was used to confirm the identities of the AMP analogs. Electrospray ionization was performed on a Finnigan TSQ700 triple quadrupole mass spectrometer (Finnigan MAT, San Jose, CA) fitted with a Finnigan ESI interface. Samples were introduced into the electrospray interface through an untreated fused-silica capillary with a 50 μm ID and 190 μm OD. A myoglobin and MRFA mixture was used for tuning and routine calibration of the instrument. The mixture was made in the following manner: stock solutions of myoglobin and MRFA were prepared by dissolving 0.9 mg of horse skeletal muscle myoglobin in 1 mL of a solution of 50:50 methanol: water containing 1% acetic acid, and 0.6 mg of MRFA was dissolved in 1 mL of a solution of 50:50 methanol: water containing 1% acetic acid. The calibration and tuning standard was made from the two stock solutions: 50 μL of myoglobin and 12 μL of MRFA were diluted with 538 μL of a 50:50 methanol:water solution in a polypropylene vial. The tuning mixture was infused into the mass spectrometer at a rate of 3 μL/min on a Harvard Apparatus (model 22, South Natick, MA) syringe pump.

Results

Intrinsic GTPase activity establishes a lower limit for a ligase reaction catalyzed by adenylosuccinate synthetase— In all ligase reactions catalyzed by the synthetase, the hydroxide anion should compete with IMP as an acceptor for the γ-phosphoryl group of GTP, or with other nucleophiles for enzyme-bound 6-PIMP. The product of the hydroxide reaction with 6-PIMP is IMP and P_i. Hence, whether the hydroxide anion is the direct acceptor of the γ-phosphoryl group of GTP or attacks atom C-6 of 6-PIMP the net result is the hydrolysis of GTP. Using C-18 HPLC, the concentrations of GDP and GTP were

monitored over time as a measure of GTP hydrolysis. Progress data were fit to coupled differential equations (Scheme I) using the program Dynafit (31).

$$E + GTP \underset{k_2}{\overset{k_1}{\rightleftharpoons}} E{\bullet}GTP$$

$$E + GDP \underset{k_4}{\overset{k_3}{\rightleftharpoons}} E{\bullet}GDP$$

$$E{\bullet}GTP \xrightarrow{k_{cat}} E + GDP + P_i$$

Scheme I

Only the concentrations of GDP and GTP were measured here as a function of time. (The concentrations of E, E•GTP and E•GDP were not directly measured). Hence, the five parameters of Scheme I seem hopelessly underdetermined. Other information, however, constrains the relative magnitudes of each of the elementary rate constants in Scheme I: (*i*) GTP and GDP bind with near equal affinities (K_i or K_d ~20 μM for Mg^{2+} and 5 μM for Ca^{2+}) to *E. coli* adenylosuccinate synthetase (1,2), and (*ii*) The kinetic mechanism is rapid equilibrium (32), hence, $k_{cat} << k_1$, k_2, k_3, and k_4. In fitting Scheme I to the data then, k_1, k_2, k_3, and $k_4$are fixed to values such that they are all large in comparison to k_{cat}, and such that $k_2/k_1 = k_4/k_3 = 20$ μM for Mg^{2+} or 5 μM for Ca^{2+} (1,2,33). Fits of the data to Scheme I then involve a single adjustable parameter k_{cat}, the values for which are reported in Table I. Variations in the fixed values of k_1, k_2, k_3, and k_4 over a million fold had no effect on the fitted value of k_{cat}, as long as $k_{cat} << k_1$, k_2, k_3, and k_4 and that the appropriate ratios were maintained for k_2/k_1 and k_4/k_3.

Control progress curves taken in the absence of enzyme showed only ~1% hydrolysis after 18 hrs (data not shown). The presence of enzyme (2 mg/mL) increased rates of GTP hydrolysis by at least 180-fold, so that after 5 hrs. the starting concentration of GTP fell by 50% (Fig. 1). The hydrolysis reaction is marginally faster in the presence of Ca^{2+} relative to Mg^{2+}, and IMP increases k_{cat} by approximately 30%. Adenylosuccinate synthetase also catalyzes GTP hydrolysis without metals and in the presence of 500 μM EDTA, although the observed activity was much lower (Table I). In the absence of metals (and with EDTA), the addition of IMP increased GTP hydrolysis approximately 20-fold.

Chemical mechanism of GTP hydrolysis— Rates of GTP hydrolysis differ with and without IMP for both the Mg^{2+}- and Ca^{2+}-catalyzed reactions. To test whether the increased rate in the presence of IMP was due in part to the introduction of an alternative catalytic pathway by way of the formation of 6-PIMP, the Mg^{2+} reaction was repeated in $H_2{}^{18}O$. Hydrolysis of 6-PIMP by the nucleophilic attack of the hydroxide anion on atom C-6 would result in ^{18}O-labelled IMP. An 18-hr. incubation using a 5-fold excess of GTP to IMP resulted in 50% GTP hydrolysis and yielded ~16% of the total IMP as 6-^{18}O-IMP (Fig. 2). The same reaction carried out in deionized H_2O, resulted in no ^{18}O-labeled of IMP.

Adenylosuccinate synthetase catalyzes a range of ligation reactions— Initial screening for substrate alternatives to L-aspartate was done by monitoring the time-dependent formation of an ultraviolet absorbance peak in chromatograms from analytical C-18 HPLC. High concentrations of the substrate alternative (100 mM) and enzyme (2 mg/mL), as well as and long incubation times (18 hr.) in principle allow the detection of even the slowest of reactions. No new products formed in the presence of α-amino alcohols, but all other

alternatives to L-aspartate in Table II resulted in new products in the presence of either Ca^{2+} or Mg^{2+}.

Subsequent purification of alternative products for mass spectrometry and ultraviolet absorbance analysis was done on a higher capacity DEAE column. Elution profiles on the anion exchange column for the amino acid products indicated an additional charge over that of unreacted IMP. All of the observed products have a mass numbers consistent with the attack of an amino group on the C-6 atom of 6-PIMP (Table I). Fig. 3 is an example of mass spectrometry and ultraviolet absorbance spectra for a purified alternative product. Hydroxylamine-*O*-sulfonic acid, yielded new product peaks on C-18 and DEAE HPLC with a λ_{max} of 265 nm, but mass spectrometry data were not consistent with the formation of the expected product, indicating the possibility of an unstable initial reaction product, or the reaction of an impurity in the hydroxylamine-*O*-sulfonic acid. In some instances mass-to-charge peaks appeared at one-half and twice that of the singly-charged product.

Extinction Coefficients of AMP Analogs— In order to more closely study the kinetics of the observed reactions, extinction coefficients were determined for the AMP analogs produced from the synthetase-catalyzed ligations with the alternative substrates. The identity and purity of products was determined by mass spectrometry. P_i contamination of the purified products was less than 1%. Products were digested with alkaline phosphatase in an essentially irreversible reaction that goes to completion. Hence, a stoichiometric amount of free P_i to AMP analog was released, providing an accurate assay of product concentration. Residual IMP from the reactions was purified and assayed in a like manner, and the determined extinction coefficient agreed with the literature value to within less than 1% error. Extinction coefficient values are listed in Table II.

Glycine Reaction Kinetics— Initial studies indicated the following order of activities for the amino acid substrates: glycine>alanine>serine. As glycine reacted the most rapidly, further studies focused on its reaction by initial velocity kinetics. Substantial amounts of enzyme (515 μg/mL) were used, and the concentration of glycine was assayed up to 1200 mM (Fig. 4). Velocities declined at higher concentrations of glycine, presumably due to ionic strength effects or perhaps substrate inhibition (data not shown). Kinetic parameters derived from fits to the data using Mg^{2+} and Ca^{2+} as activators are in Table III. The K_m for glycine is virtually the same regardless of whether the cation is Mg^{2+} or Ca^{2+}, while the k_{cat} for the latter is about 50% greater. The dependency of glycine on the metal activator differs substantially from that of L-aspartate. Although L-aspartate has a lower K_m for the Ca^{2+}-activated enzyme than that for the Mg^{2+}-activated system, the rate of catalysis supported by Ca^{2+} is 7-fold lower than for Mg^{2+}.

Acetate inhibition of the adenylosuccinate synthetase reaction is competitive with respect to L-aspartate for both the Mg^{2+}- and Ca^{2+}-catalyzed reactions, as determined by model discrimination analysis in Dynafit (31). Acetate chelates divalent cations, but based on the formation constants for $[MgOAc]^-$ and $[CaOAc]^-$, the levels of free Mg^{2+} or Ca^{2+} never fell below 3 mM in assays. The kinetic data and models are shown in Fig 5. and the fit parameters for acetate inhibtion are in Table III.

Discussion

The proposed chemical mechanism for adenylosuccinate synthetases is a two-step reaction in which 6-PIMP forms first from the attack of the 6-oxyanion of IMP on the γ-phosphoryl group of GTP, followed by the nucleophilic attack of L-aspartate on the cationic form of 6-

PIMP (Figs. 6 and 7). Crystallographic structures suggest the possible protonation of atom N-1 of 6-PIMP, which would result in the localization of positive charge on the 6-carbon atom. In principle, the cationic form of 6-PIMP should react with any reasonable nucleophile in a simple bimolecular reaction, even in the absence an initial Michaelis complex. The reaction with hydroxylamine, as suggested by Gorrell *et al.* (34), may in fact be a bimolecular reaction between hydroxylamine and the activated 6-PIMP-enzyme complex. The observed nonlinear response at high concentrations of hydroxylamine, which is consistent with the formation of a Michaelis complex, may be the consequence of inhibition by the chloride anion, which is present at a fixed proportion to hydroxylamine. Sodium and potassium chloride, for instance cause significant inhibition of the L-aspartate catalyzed reaction at a concentration of 0.5 M where "saturation effects" are observed (E. Underbakke and R. B. Honzatko, unpublished data). The hydroxyl group of hydroxylamine is not essential to reactivity as methoxylamine and ethoxylamine support product formation (Table II). Only the formation of a hydrogen bond between the amine group of hydroxylamine and backbone carbonyl 38 could support the formation of a productive Michaelis complex, an interaction proposed for the α-amino group of L-aspartate on basis of structural investigations (Fig. 5) (17,19). On the other hand, carboxymethoxylamine could form an initial Michaelis complex through interactions involving its carboxyl group and the L-aspartate binding pocket.

Even though in principle any nucleophile could collide and react with the activated 6-PIMP-enzyme complex, all potential substrates of the synthetase are in competition with the hydroxide anion. A nucleophile will be a substrate for adenylosuccinate synthetase provided that its k_{cat} value exceeds that for GTP hydrolysis. The synthetase catalyzes at least two

chemical mechanisms of GTP hydrolysis: the hydroxide anion directly hydrolyzes enzyme-bound GTP by attacking its γ- and/or β-phosphoryl group or indirectly hydrolyzes GTP by attacking atom C-6 of 6-PIMP. Evidence of the former mechanism comes from the catalysis of GTP hydrolysis in the absence of IMP, whereas evidence of the latter mechanism comes from the incorporation of ^{18}O at the 6-position of IMP. Incomplete labeling of atom O-6 of IMP is not surprising. The concentration of ^{18}O labeled water in the reaction mixture (after the addition of all reaction constituents) is 75%. Hence, at best only three of four hydrolysis events involving 6-PIMP would result in labeling. Correcting for isotope dilution, at least 21% of the IMP becomes labeled during the hydrolysis of 50% of the GTP. If IMP remains bound to the enzyme for several hydrolysis events, or if labeled IMP re-associates with the enzyme, then the indirect mechanism could be the dominant pathway.

The enzyme is almost equally proficient in the hydrolysis of GTP using Mg^{2+} or Ca^{2+} in the absence or presence of IMP. Moreover, IMP in the absence of divalent cation supports the hydrolysis of GTP at a rate comparable to that in the presence of Ca^{2+} or Mg^{2+}. Although Mg^{2+} is considered an essential cation activator of adenylosuccinate synthetase (1,2,15), under conditions of saturating IMP, GTP and L-aspartate, the enzyme is probably active, albeit the level of activity may be no more than 0.01% of that in the presence of saturating metal. Indeed, co-crystallization of the synthetase with IMP, GTP and L-aspartate and 1 mM EDTA results in a complex of SAMP and GDP at the active site (C. V. Iancu and R. B. Honzatko, unpublished data).

Evidently, the α-carboxyl group plays a critical role in making a nucleophile into a viable substrate for the synthetase. α-Amino alcohols of glycine, alanine and serine participate in no apparent reaction, but the corresponding amino acids react to form new

products. The glycine-driven reaction (and presumably the others as well) exhibits saturation kinetics. (Unlike the hydroxylamine, which is used as its acid chloride, no chloride is associated with the free-acid form of glycine used in the assays here). The K_m for glycine is approximately two thousand-fold higher than that for L-aspartate, and although its k_{cat} is only ~1% of that of L-aspartate reaction, it is still 20-fold faster than the rate of GTP hydrolysis. Most striking in regard to the glycine-driven reaction is its insensitivity to the type of divalent cation. L-Aspartate has significantly lower values for K_m and k_{cat} when the divalent cation is Ca^{2+} instead of Mg^{2+}, whereas the kinetic parameters of glycine show no variation with metal type. Crystallographic structures of the synthetase complexes of adenylosuccinate and of hadacidin imply the direct coordination of the α-carboxyl group of L-aspartate to the catalytic metal, thereby suggesting a basis for the observed metal sensitivity in the kinetics of L-aspartate (17–21). The absence of metal sensitivity in the kinetic parameters of glycine implies at best a weak interaction between its α-carboxyl group and the catalytic metal. Arg305 of the *E. coli* synthetase also hydrogen bonds to the α-carboxyl group of L-aspartate (Fig. 8) (19,35), and we suggest that glycine succeeds as a substrate because of this putative interaction with Arg305. Indeed, the charge neutralization of the α-carboxyl group of glycine caused by an interaction with Arg305, should lower the pK_a of its α-amino group and enhance its reactivity.

Why is L-aspartate far superior to other α-amino acids as a substrate of the synthetase? One possibility is that the interactions at the β-carboxyl group provide sufficient free energy to compress the α-carboxyl group of L-aspartate into the coordination sphere of the catalytic Mg^{2+}. The coordination sphere for Ca^{2+} is larger than that of Mg^{2+}, so that the α-carboxyl group of L-aspartate experiences less steric crowding in the Ca^{2+}-activated

enzyme. If this view is correct, then acetate and/or succinate may be far more effective as inhibitors of the Ca^{2+}-activated enzyme, than the Mg^{2+}-activated system. Acetate inhibits the Ca^{2+}-activated enzyme no more effectively that the Mg^{2+}-activated system, but the observed competitive mechanism of inhibition may be a consequence of the binding of acetate to the pocket for the β-carboxyl group of L-aspartate. In crystal structure where the total concentration of acetate is approximately 400 mM, electron density for a disordered water molecule or an acetate anion is in the binding pocket for the β-carboxyl group of L-aspartate, but no electron density is present for a metal-coordinated acetate.

The ability of adenylosuccinate synthetase to catalyze reactions with hydroxide, derivatives of hydroxylamine and several amino acids is consistent with the existence of an activated intermediate of 6-PIMP. These reactions seem not to depend on the type of divalent metal cation, and could proceed even in the absence of a divalent metal cation. The catalyzed reactions may not be metal independent, however, as the investigations here make no effort to limit monovalent cations (primarily Na^{+}), which are introduced as counterions to substrates. Adenylosuccinate synthetase may find utility in the synthesis of N-6 substituted derivatives of adenine nucleotides, which have been used as probes in cell signaling cascades (36,37). An alternative organic synthetic pathway employs the nucleophilic displacement of 6-chloropurine ribonucleotide by the α-amino group of an amino acid (38–40). The organic synthetic protocol works for the synthesis of a variety of N-6 substituted derivatives (38), but requires 6-chloropurine ribonucleotide as a starting material. The synthetase-mediated reaction in many respects is similar in its fundamental chemistry (nucleophilic displacement of phosphate instead of chloride), but requires IMP and GTP, both of which are readily

available. Hence, in specific instances the synthetase may be a viable alternative to traditional synthetic routes for the synthesis of 6-substituted adenines.

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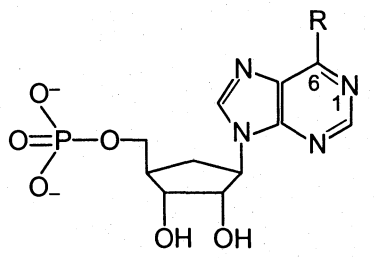
Hydrolysis Reaction	k_{cat} (s^{-1})	Reaction velocity ($nM*s^{-1}$)
Enzyme present:		
Mg^{2+}	0.00065(3)	28(1)
Mg^{2+}/IMP	0.00085(3)	37(1)
Ca^{2+}	0.00070(2)	30(1)
Ca^{2+}/IMP	0.00095(3)	41(1)
EDTA	0.00005(2)	2(1)
EDTA/IMP	0.00090(4)	39(2)
Enzyme absent:		
Mg^{2+}	–	0.08(1)
Mg^{2+}/IMP	–	0.12(1)
No additions to GTP	–	0.12(1)

Table I. Rates of GTP hydrolysis. k_{cat} values for GTP hydrolysis come from fitting the coupled differential equations based on Scheme I, subject to the constraints described in the Results section. Reaction velocities in the presence of enzyme are $k_{cat}*[E]$, where [E] represents the concentration of enzyme used in the experiment. The velocities of reactions in the absence of enzyme are the slopes of linear progress curves. Numbers in parentheses represent the standard deviation in the last significant digit of the associated value. GTP is present initially at 1 mM in all reactions. Metals, IMP, and EDTA, when present, are at concentrations of 5, 1 and 0.5 mM.



Reactant	R, position 6 substituent	Retention time on C-18 (min)	Mass Spec. peaks	λ_{max} (nm)	ϵ_{max} ($M^{-1}\cdot cm^{-1}$) $x10^{-3}$
Hydroxylamine[c]	HO–NH–	7.6	362	266	16.0 (6)
Methoxylamine[d]	CH_3–O–NH–	11.4	376	267	16.2 (8)
Ethoxylamine[e]	CH_3CH_2–O–NH–	14.8	390	267	17.0 (7)
Carboxymethoxyl amine[f]	$^-OOCCH_2$–O–NH–	13.5	420	268	17.4(8)
Hydroxylamine-*O*-sulfonate	^-O_3S–O–NH–	8.7	---[a]	--[a]	---[a]
Glycine[g]	$^-OOCCH_2$–NH–	14.6	404	266	17.5 (4)
L-Alanine[h]	$^-OOCCH_2CH(CH_3)$–NH–	17.2	418	267	17.0 (9)
L-Serine[i]	$^-OOCCH_2CH(CH_2OH)$–NH–	13.9	434	267	17.3 (8)
L-Glutamate[j]	$^-OOCCH_2CH(CH_2CH_2COO^-)$–NH–	15.6	476	267	17.1(9)
L-Aspartate[k]	$^-OOCCH_2CH(CH_2COO^-)$–NH–	15.2	462	267	16.9[b]

Table II. Results of the Screen for Alternative Substrates. (legend continued on next page)

Table II. Results of the Screen for Alternative Substrates. New peaks from C-18 reverse-phase chromatography indicated a successful reaction. Purified products were analyzed by mass spectrometry and ultraviolet absorbance spectroscopy. Major mass spec. peaks correspond to $[MH]^-$. All products have the common nucleotide structure given in the header, with the symbol *R* representing the structurally variant group. No reaction was observed for glycinol, alaninol, or serinol. [a]Reaction product not stable. [b]Extinction coefficient comes from reference (15). [c]{(2*R*,3*S*,4*R*,5*R*)-3,4-dihydroxy-5-[6-(hydroxyamino)-9*H*-purin-9-yl]tetrahydrofuran-2-yl}methyl dihydrogen phosphate. [d]{(2*R*,3*S*,4*R*,5*R*)-3,4-dihydroxy-5-[6-(methoxyamino)-9*H*-purin-9-yl]tetrahydrofuran-2-yl}methyl dihydrogen phosphate. [e]{(2*R*,3*S*,4*R*,5*R*)-3,4-dihydroxy-5-[6-(ethoxyamino)-9*H*-purin-9-yl]tetrahydrofuran-2-yl}methyl dihydrogen phosphate. [f]{[(9-{(2*R*,3*S*,4*R*,5*R*)-3,4-dihydroxy-5-[(phosphonooxy)methyl]tetrahydrofuran-2-yl}-9*H*-purin-6-yl)amino]oxy}acetic acid. [g]2-[(9-{(2*R*,3*S*,4*R*,5*R*)-3,4-dihydroxy-5-[(phosphonooxy)methyl]tetrahydrofuran-2-yl}-9*H*-purin-6-yl)amino]acetic acid. [h](2*S*)-2-[(9-{(2*R*,3*S*,4*R*,5*R*)-3,4-dihydroxy-5-[(phosphonooxy)methyl]tetrahydrofuran-2-yl}-9*H*-purin-6-yl)amino]propanoic acid. [i](2*S*)-2-[(9-{(2*R*,3*S*,4*R*,5*R*)-3,4-dihydroxy-5-[(phosphonooxy)methyl]tetrahydrofuran-2-yl}-9*H*-purin-6-yl)amino]-3-hydroxypropanoic acid. [j](2*S*)-2-[(9-{(2*R*,3*S*,4*R*,5*R*)-3,4-dihydroxy-5-[(phosphonooxy)methyl]tetrahydrofuran-2-yl}-9*H*-purin-6-yl)amino]pentanedioic acid. [k](2*S*)-2-[(9-{(2*R*,3*S*,4*R*,5*R*)-3,4-dihydroxy-5-[(phosphonooxy)methyl]tetrahydrofuran-2-yl}-9*H*-purin-6-yl)amino]succinic acid

Kinetic parameter	Mg^{2+}-activated enzyme	Ca^{2+}-activated enzyme
K_m-L-aspartate (μM)	140 ± 20	53 ± 6
k_{cat}-L-aspartate (s^{-1})	1.03 ± 0.04	0.16 ± 0.01
K_m-glycine (mM)	440 ± 40	410 ± 40
k_{cat}-glycine (s^{-1})	0.015 ± 0.001	0.023 ± 0.001
K_i-acetate (mM)	31 ± 4	77 ± 8

Table III. Kinetic parameters for Mg^{2+}- and Ca^{2+}-activated synthetases. Conditions for assays are provided in the Experimental section. K_m-L-aspartate and K_m-glycine are the Michaelis constants for L-aspartate and glycine, respectively. Acetate is a competitive inhibitor with respect to L-aspartate.

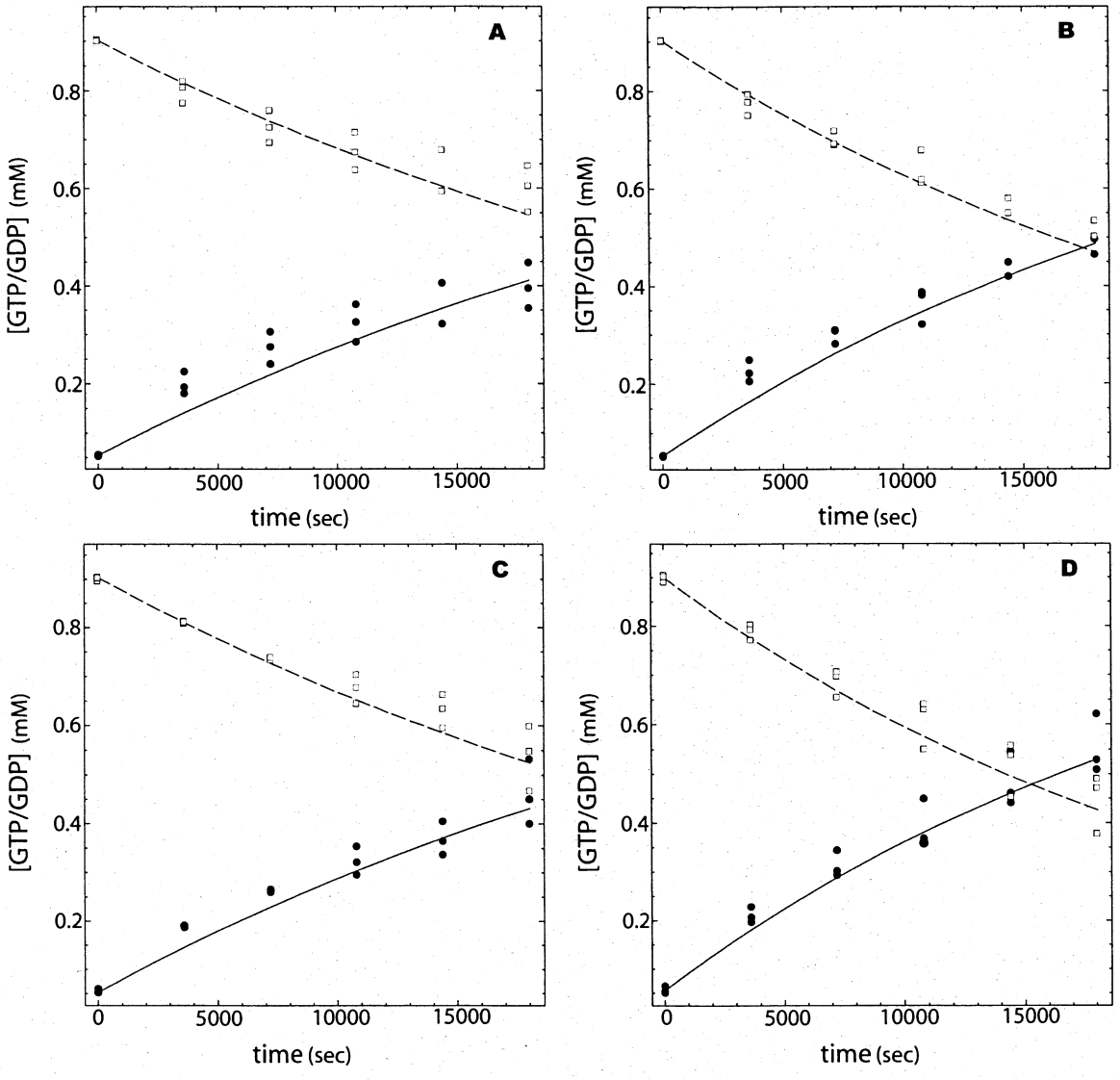


Fig. 1. Progress data for GTP hydrolysis. Incubations for both the Mg^{2+} and Ca^{2+} catalyzed reactions were done at 37 °C. Aliquots were taken each hour for five hours and analyzed by C-18 HPLC to determine concentrations of GDP (●) and GTP (▫). **A**) Mg^{2+} mediated hydrolysis with no IMP, **B**) Mg^{2+} mediated hydrolysis in the presence of IMP, **C**) Ca^{2+} mediated hydrolysis with no IMP, **D**) Ca^{2+} mediated hydrolysis in the presence of IMP.

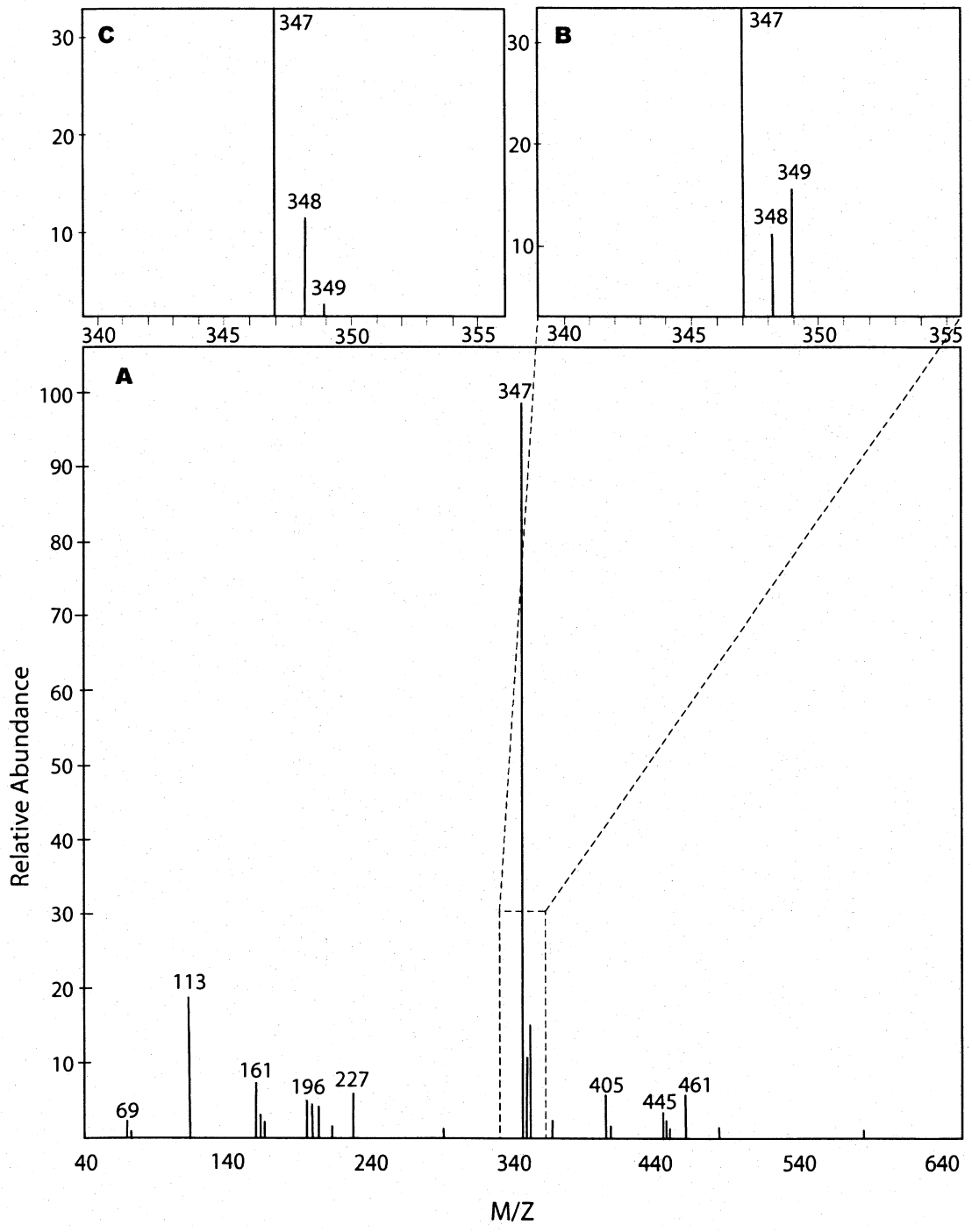


Fig. 2. Mass spectrum analysis of the ^{18}O exchange experiment. **A**) mass spectrum of IMP isolated from the isotope incubation, **B**) zoom view of IMP peaks from isotope incubation, **C**) zoom view of IMP peak from control incubation. The molecular weight of IMP is 348 and the parent peak at 347 represents $[MH]^-$. The peak at 348 represents the ^{13}C isotopic forms, and the peak at 349 represents the +2 exchange of ^{18}O into IMP.

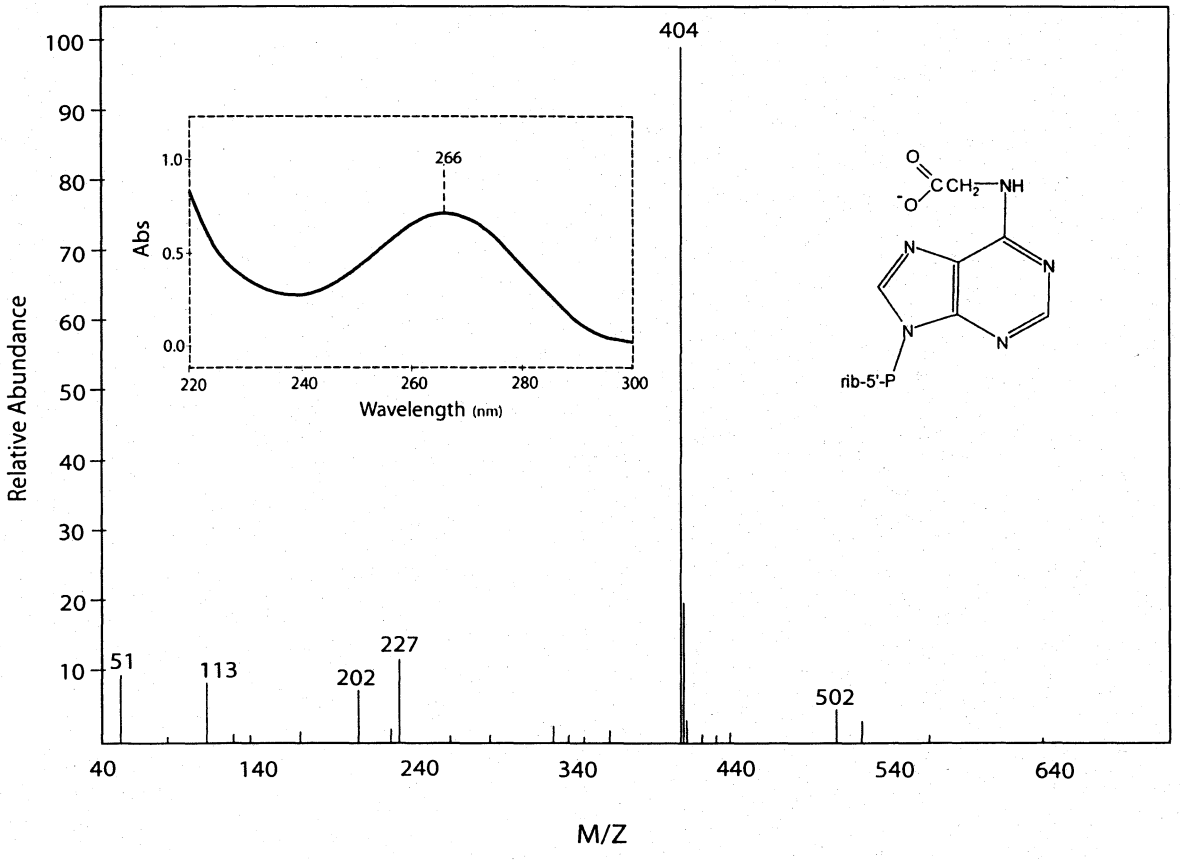


Fig. 3. Mass spectrum of the glycine product. The spectrum was obtained by negative ion mode ESI. The calculated molecular weight is 405 Da and the parent peak at 404 represents $[MH_2]^-$. Inset is a UV spectrum of the same sample.

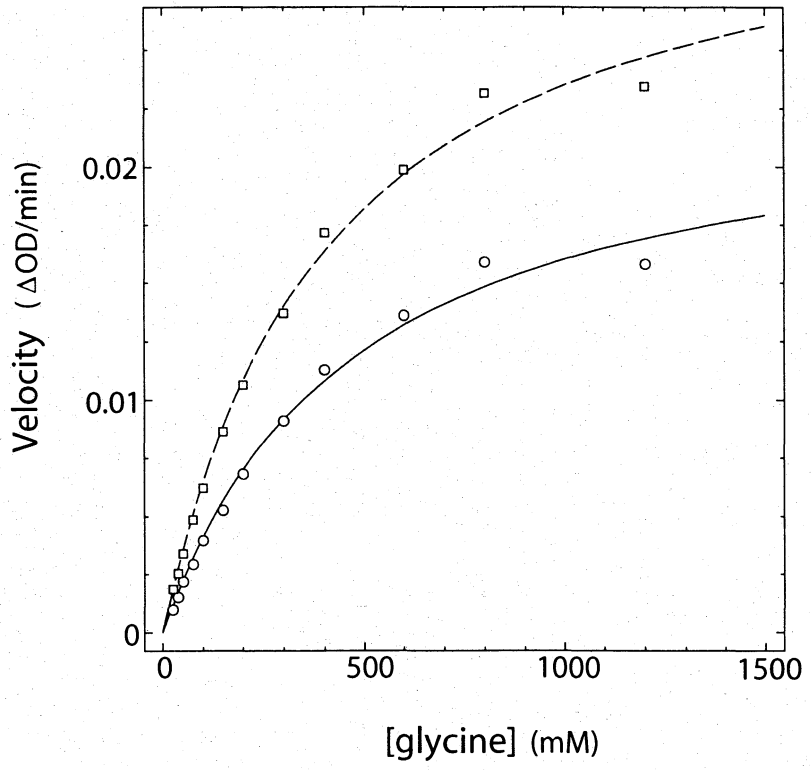


Fig. 4. Determination of Michaelis constants for the glycine reaction. Initial velocity data for the Mg^{2+} (○) and Ca^{2+} (□) catalyzed reactions. Fitting to the Michaelis-Menten equation was done using Dynafit.

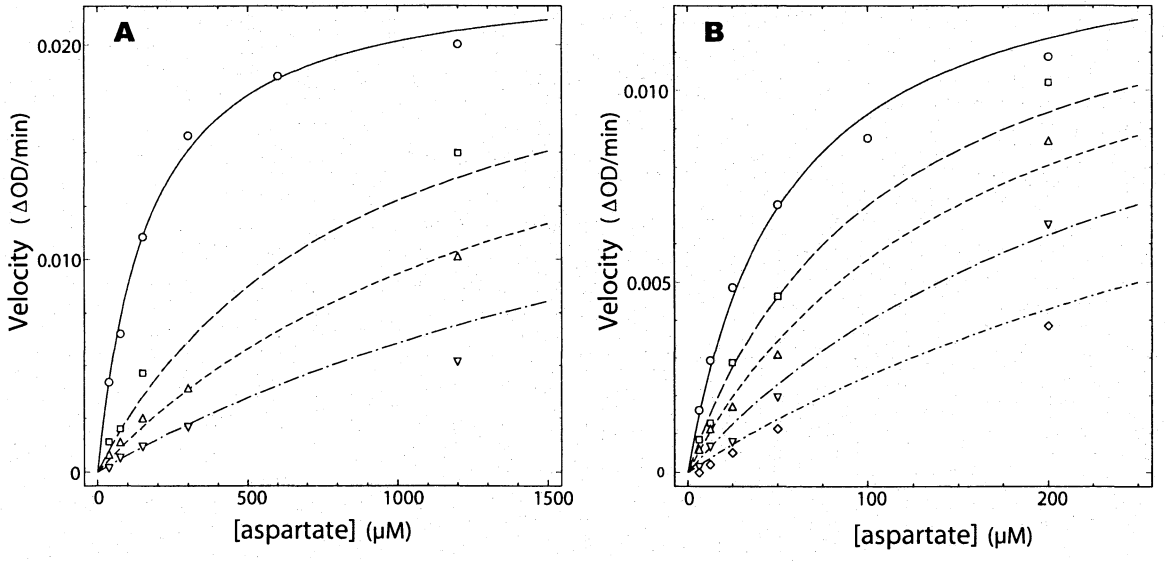


Fig. 5. Acetate inhibition of the AMPSase reaction. Assays were done with no inhibitor (○), or 75mM (▫), 150 mM (△), 300mM (▽), and 600mM (◇) acetate for the **A**) Mg^{2+} and **B**) Ca^{2+} catalyzed reactions. Nonlinear fits to a rapid-equilibrium competitive mechanism were done using Dynafit.

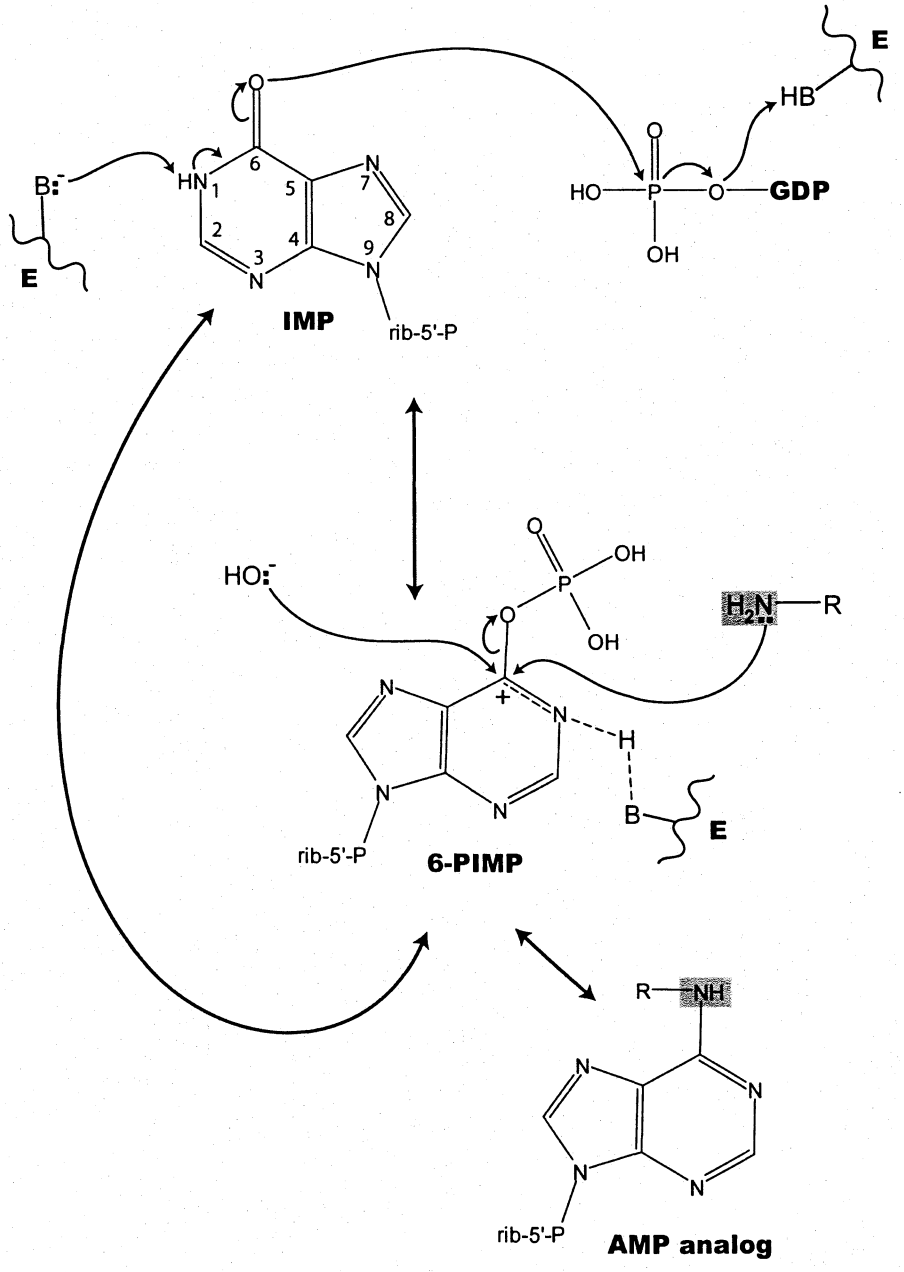


Fig. 6. The 6-phosphoryl-IMP mechanism. Enzymatic activation of IMP for nucleophilic attack leads to formation of 6-PIMP. This intermediate is then open to attack by a variety of amino-nucleophiles and to hydrolysis to IMP.

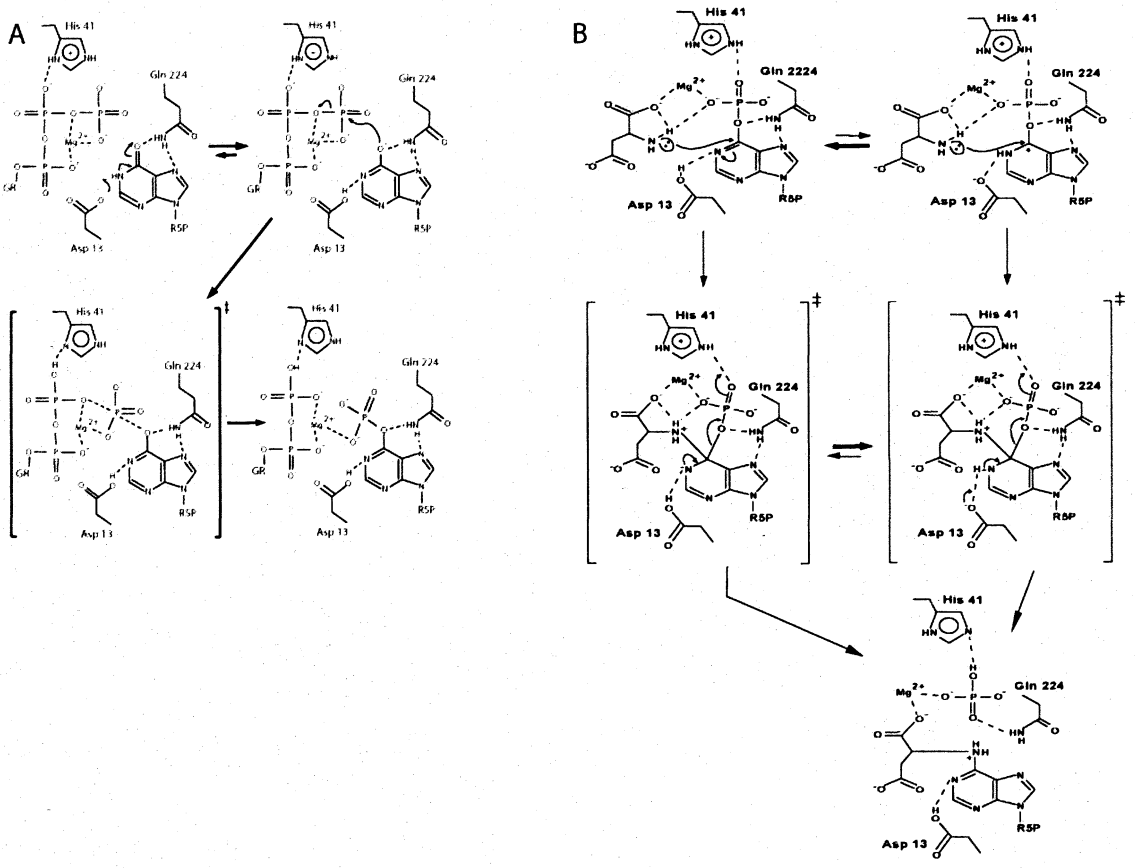


Fig. 7. More detailed 6-PIMP mechanism. **A**) The first step of the mechanism, where IMP attacks the γ-phosphoryl of GTP. **B**) The second step of the mechanism, where aspartate displaces the 6-phosphoryl of 6-PIMP. Putative roles for residues important for catalysis are shown.

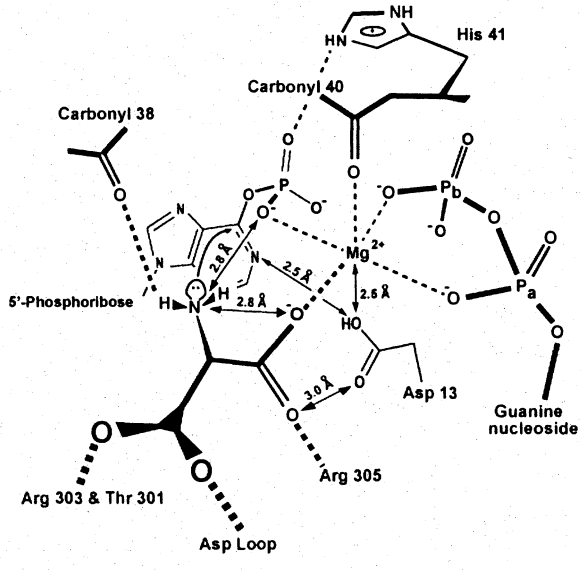


Fig. 8. View of the crowded Mg^{2+} coordination sphere, as modeled from crystal structures. Critical residues for catalysis (such as Asp13 and His41) are shown, as well as the bound substrates. Mg^{2+} (and other divalent cations) putatively coordinates to the phosphoryl groups of GTP, and to the phosphoryl group of 6-PIMP once it is formed. As well, the α-carboxyl group of aspartate likely interacts with the metal as well, destabilizing the zwitterion and activating the amino group for nucleophilic attack on 6-PIMP.

CHAPTER 3: GENERAL CONCLUSIONS

Summary

The catalytic promiscuity of adenylosuccinate synthetase is of great value for several reasons. Alternate substrates can reveal functional determinants of catalysis that are not always apparent even from multiple crystal structures. Broad reactivity suggests the possibility of a high-energy intermediate of the reaction that could undergo a variety of chemical reactions. The products resulting from these reactions could have profound pharmacological effects, or be useful tools in the study of other systems.

Understanding the determinants of specificity of adenylosuccinate synthetase for aspartate has lagged in relation to its other substrates. This is due in part to the lack of structural information. A crystal structure of the acidic mouse isozyme with aspartate bound to the active site (as yet unpublished) was solved only recently. Studies presented in this thesis present an approach, different from crystallography, to understanding how the synthetase recognizes aspartate and analogs of aspartate. All previously known substrates of adenylosuccinate synthetase, save for hydroxylamine, contained both an α-carboxyl group, and a β-carboxyl group (or a close approximation thereof). It was assumed that the α-carboxyl played a role in orientation of the substrate through binding interactions with the enzyme and the bound metal, but that this role was not crucial for catalysis. Moreover, the β-carboxyl was considered crucial, in that it provided the "push" necessary to promote catalysis. Experimental data presented in this thesis demonstrate that the β-carboxyl group is not absolutely necessary, while the role of the α-carboxyl

group is both different and more important than previously thought. Studies with aspartate would not have revealed these behaviors.

In terms of a bigger picture, some of the products of the alternate reactions catalyzed by adenylosuccinate synthetase could be drug candidates. One product, the result of a reaction with β-cysteine sulfinate, has been shown to exhibit an important physiological role by promoting heavy metal tolerance in the fission yeast *Schizosaccharomyces pombe.* Recent use of bulky 6-amino substituted AMP analogs in elucidating cell-signaling pathways provides an additional impetus for harnessing the promiscuity of adenylosuccinate synthetase. The enzyme represents a way to produce these analogs more efficiently and under milder conditions than for a typical organic synthesis.

Further study of these reactions, utilizing crystallography and site-directed mutagenesis, could result in mutants that are even more efficient catalysts to produce these novel compounds. Substituting Arg303 and Arg305, residues that bind the carboxyl groups of aspartate, for example, may increase reactivity toward the α-amino alcohols and other non-carboxyl containing substrates that don't react with the wild-type enzyme.

In addition, many potential substrates have limited solubility, which is a major concern for substrates with K_m values in the hundreds of millimolar. Surface polar residues of the synthetase could be modified to optimize the enzyme for a more hydrophobic environment. This would have the added benefit of reducing the effective concentrations of water and hydroxide, which in turn would reduce the limiting GTP hydrolysis reaction.

The intrinsic GTPase activity of adenylosuccinate synthetase presented an opportunity to demonstrate formation of the 6-PIMP intermediate by the enzyme. The exchange of $^{18}OH^-$ into IMP bolsters an already large body of evidence for formation of the intermediate already obtained from isotope exchange experiments and from crystal structures.

In all, the studies presented in this thesis embody a unique examination of the properties of adenylosuccinate synthetase. This work has demonstrated some unexpected phenomena, and at the very least, a new and powerful tool for the synthesis of AMP analogs.

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