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FUNGAL ASPARTATE KINASE MECHANISM AND INHIBITION

By

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FUNGAL ASPARTATE KINASE MECHANISM AND INHIBITION

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ABSTRACT

Aspartate kinase (AK) from *Saccharomyces cerevisiae* (AK_{Sc}) catalyzes the first step in the aspartate pathway responsible for biosynthesis of L-threonine, L-isoleucine, and L-methionine in fungi. Little was known about amino acids important for AK_{Sc} substrate binding and catalysis. Hypotheses about important amino acids were tested using site directed mutagenesis to substitute these amino acids with others having different properties. Steady state kinetic parameters and pH titrations of the variant enzymes showed AK_{Sc}-K18 and H292 to be important for binding and catalysis.

Little was known about how the *S. cerevisiae* aspartate pathway kinases, AK_{Sc} and homoserine kinase (HSK_{Sc}), catalyze the transfer of the γ-phosphate from adenosine triphosphate (ATP) to L-aspartate or L-homoserine, respectively. Two transfer paths are possible as are a range of mechanisms, with two extremes. Both kinases were shown to directly transfer the γ-phosphate from ATP to the amino acid. Experimental evidence was consistent with an associative mechanism of phosphoryl transfer but did not rule out a dissociative mechanism.

AK_{Sc} and HSK_{Sc} are good targets for inhibitors because they catalyze phosphoryl transfers between very similar substrates. An attempt was made to rationally design inhibitors to these kinases by linking the substrates with a variable length linker to create bisubstrate compounds. This strategy failed inhibit AK_{Sc} and HSK_{Sc}, however, one of the bisubstrate compounds was a good inhibitor of AKIII from *Escherichia coli*.

Inhibition of any of the enzymes in the aspartate pathway would lead to reduced production of amino acids. A pathway assay was optimized to allow screening of chemical libraries in the hope of identifying inhibitors to the first four pathway enzymes. A high throughput screen of 1,000 compounds identified two compounds capable of inhibiting the assay, one of which was the best inhibitor identified for AK_{Sc}.

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Thanks goes out to my close family for being there for me. I have no doubt that I wouldn't have written this without you.

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For my mother

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AAK	amino acid kinase
ABC	adenosine triphosphate binding cassette
ACT	aspartate kinase, chorismate mutase, and TyrA (prephenate dehydrogenase)
ADP	adenosine diphosphate
AK	aspartate kinase
$AKIII_{Ec}$	*Escherichia coli* aspartate kinase III
AK_{Sc}	*Saccharomyces cerevisiae* aspartate kinase
$AKI\text{-}HSDI_{Ec}$	*Escherichia coli* aspartate kinase I – homoserine dehydrogenase I
$AKII\text{-}HSDII_{Ec}$	*Escherichia coli* aspartate kinase II – homoserine dehydrogenase II
ASD	aspartate semialdehyde dehydrogenase
ASD_{Ec}	*Escherichia coli* aspartate semialdehyde dehydrogenase
ASD_{Sc}	*Saccharomyces cerevisiae* aspartate semialdehyde dehydrogenase
ATP	adenosine triphosphate
CDR	*Candida* drug resistance
DMSO	dimethyl sulfoxide
DTNB	5,5'dithiobis-(2-nitrobenzoic acid)
EDTA	ethylenediaminetetraacetic acid
GHMP	galactokinase, homoserine kinase, mevalonate kinase, and phosphomevalonate kinase
HEPES	4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid

HON	(*S*)-2-amino-4-oxo-5-hydroxypentanoic acid
HSD	homoserine dehydrogenase
HSAT	homoserine *O*-acetyl transferase
$HSAT_{Hi}$	*Haemophilus influenzae* homoserine *O*-acetyl transferase
$HSAT_{Sp}$	*Schizosaccharomyces pombe* homoserine *O*-acetyl transferase
HSD_{Sc}	*Saccharomyces cerevisiae* homoserine dehydrogenase
HSK	homoserine kinase
HSK_{Ec}	*Escherichia coli* homoserine kinase
HSK_{Mj}	*Methanococcus jannaschii* homoserine kinase
HSK_{Sc}	*Saccharomyces cerevisiae* homoserine kinase
HSK_{Sp}	*Schizosaccharomyces pombe* homoserine kinase
HTS	homoserine transsuccinylase
HTS_{Ec}	*Escherichia coli* homoserine transsuccinylase
IPTG	isopropyl β-D-1-thiogalactopyranoside
L-APPA	L-2-amino-5-phosphono-3-*cis*-pentenoic acid
MSF	major facilitator superfamily
NAD^+	oxidized nicotinamide adenine dinucleotide
NADH	reduced nicotinamide adenine dinucleotide
$NADP^+$	oxidized nicotinamide adenine dinucleotide phosphate
NADPH	reduced nicotinamide adenine dinucleotide phosphate
$NAGK_{Ec}$	*Escherichia coli N*-acetylglutamate kinase

PIX	positional isotope exchange
PK-LDH	pyruvate kinase – lactate dehydrogenase
PMSF	phenylmethylsulfonyl fluoride
RI-331	(*S*)-2-amino-4-oxo-5-hydroxypentanoic acid
SAM	*S*-adenosylmethionine
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate – polyacrylamide gel electrophoresis
S_N1	unimolecular nucleophilic substitution
S_N2	bimolecular nucleophilic substitution
TS	threonine synthase
TS_{At}	*Arabidopsis thaliana* threonine synthase
TS_{Ec}	*Escherichia coli* threonine synthase
TS_{Sc}	*Saccharomyces cerevisiae* threonine synthase
UMP	uridine monophosphate

Chapter 1

Introduction

1.1. The Need for Antifungal Agents

Fungi impact human activity, either directly due to medical problems, or indirectly through effects on our ability to produce food and build infrastructure. To reduce the costs inflicted by fungi, humans have developed ways to battle fungi through antifungal agents. This creates a strong selective pressure on fungi to develop resistance to these agents, and with time, fungi have developed such resistances.

The possible direct medical impacts of fungi on humans are many, but are usually restricted to humans that are unwell because the immune system of healthy individuals is usually sufficient to prevent fungal infections. As a result, fungal infections of humans are generally opportunistic in nature. For example, patients with neutropenia are at increased risk of infections caused by *Candida* and *Aspergillus* which are the most common pathogens, as well as to less common infections caused by *Trichosporon* and *Fusarium* (Armstrong 1993). Many of the *Candida* infections of neutropenic patients occur through intravenous or bladder catheters which provide an easy way past the skin barrier. These invasive infections have high mortality rates, for example *Candida* bloodstream infections kill up to 40% of those infected while invasive *Aspergillus* infections are even higher at 50% (Masia Canuto and Gutierrez Rodero 2002). *Candida* overgrowth in the mouth and throat (thrush) is the most common fungal infection of humans (Akpan and Morgan 2002). Such infections lead to significant discomfort which in turn, can result in poor nutrition. Even with treatment, these infections are slow to

cure and may require hospital stays (Akpan and Morgan 2002). *Aspergillus* infections usually start in the lung and can be extremely persistent sometimes requiring surgical removal of the lung lesion (Armstrong 1993; Graybill 1996).

Clinicians regularly encounter 30 fungal species which can cause serious systemic infections in humans, while upwards of 200 have been documented to be pathogenic (Ellis 2002). However, a worrisome prospect is that the list of fungal pathogens is growing due to increasing populations of patients at risk of opportunistic infections as other areas of medicine improve their ability to keep patients alive (Sternberg 1994). For example, better treatments for cancer and AIDS mean there are more people alive with reduced immune system function. As well, there are increasing numbers of people living with organ transplants that are taking anti-rejection drugs to prevent rejection of the new organ by attenuating the immune system. These increases in susceptible people have allowed a mushrooming of the total number of fungal infections, for example, *Candida* is now the fourth leading cause of nosocomial bloodstream infections (Edmond et al. 1999). One would expect the number of susceptible people to remain constant or increase in the future (Georgopapadakou and Walsh 1996). The increase is also due to humans inhabiting more areas of earth, and by pushing back the edges of forests and marshlands we are being exposed and re-exposed to greater numbers of fungal species. Global travel quickly allows newly encountered fungi to be disseminated to populations around the world, where susceptible people can become infected. Thus there is more opportunity for previously benign fungi to find new niches in which to live.

There are several other consequences beyond the medically important detrimental consequences of fungal interactions with humans. Fungi are major players in the global carbon cycle by taking carbon found in dead or dying trees, releasing it as carbon dioxide in the atmosphere where it is redistributed globally to be fixed by photosynthesis back into a biologically useful form. The participation of fungi in the carbon cycle only becomes problematic to humans when the wood in human structures is recycled. Fungi may also sicken humans indirectly by growing in buildings. For example, *Stachybotrys chartarum* has been implicated as a causative agent in mold-induced building-related illness due to its ability to produce mycotoxins, however, the link between growth in buildings and causing disease is still debatable (Kuhn and Ghannoum 2003).

A more significant indirect problem is the fungal infection of crops, both before and after harvest. Pre-harvest fungal infections are those that occur in the field and cause reductions in yield. A classic example is the Irish Potato Famine of the 1840s which has been vividly described (Hudler 1998), where the potato crop, on which the vast majority of the Irish population subsisted, was decimated by *Phytophthora infestans* for several years. It should be noted that *Phytophthora infestans* is no longer considered a true fungus, but is an Oomycete which falls into the Kingdom of Protoctista or Chromista (Fry and Goodwin 1997) and is related to diatoms and brown algae (Judelson 1997). Fungi have caused great losses in many crops in the field including grains such as wheat and corn; fruit such as grapes, apples, and tomatoes; and vegetables such as potatoes,

onions, and celery. Fungal diseases of trees have had very large ecological impacts. A prime example is Chestnut Blight caused by *Cryphonectria parasitica*, which prevents water transport from roots to leaves. This disease killed all of the American Chestnut trees in North America in the first half of the twentieth century (Hudler 1998). Another important example is Dutch Elm Disease caused by *Ophiostoma ulmi* which plugs the cells that carry water from the roots to the leaves, eventually killing the tree, and since first being discovered in 1940 it has killed hundreds of thousands of trees in North America (Hudler 1998).

Post-harvest effects of fungi on stored foodstuffs are responsible for a large losses to the overall productivity in agriculture. In addition to directly damaging stored grains and killing stored seeds, fungi can affect the quality and appearance of stored grains by discolouring the grain and/or introducing foul odours and tastes, thus decreasing selling prices. Furthermore, fungi can increase the temperature within grain silos due to the heat of respiration which can lead to spontaneous combustion. Lastly, during storage fungi can also produce mycotoxins which are toxic to animals (Muir and White 1999). In Canada, *Penicillium* and *Aspergillus* are the most common fungi found in stored wheat (Muir and White 1999).

Mycotoxin production by fungi poses a serious health problem for humans in that many fungi that infect crops produce these metabolites. Mycotoxins are secondary metabolites produced by filamentous fungi and fall into several groups, including:

aflatoxins, trichothecenes, and ergot alkaloids, with total numbers of isolated compounds greater than 300 (Hussein and Brasel 2001). The effects of these toxins on humans include carcinogenesis, vomiting, convulsions, and hallucinations (Hussein and Brasel 2001). Many effects are found in livestock that consume contaminated grasses and grains, thus the effects of mycotoxins can impact humans in many ways. Two mycotoxins have even been listed on the U.S. "Select Agents List" which identifies potential biological and chemical agents which could be used by terrorists, and outlines who is permitted to possess them (Desjardins 2003).

As will be discussed in the following sections, there are few antifungal agents used clinically and those in use are frequently ineffective for certain infections, sometimes toxic, and at times very expensive (Walsh et al. 2000). The following sections also demonstrate that there are very few fungal enzymes or structures that are the target of the currently used antifungal agents. Cross resistance between currently used antifungals exists and more could develop. For these reasons, new antifungals which target new enzymes or structures are greatly needed.

1.2. Current Antifungal Agents

The polyene macrolide group of antifungal agents has two important members, amphotericin B, Table 1.1, and nystatin, which were discovered in the early 1950's (Bossche et al. 2003). Nystatin structurally differs from amphotericin B in the placement of two hydroxyl groups. Amphotericin B is considered the 'Gold Standard', against which all other antifungals are compared. The polyenes are produced by *Streptomyces* and are selectively toxic to fungal cells because they bind the ergosterol found in fungal membranes that is absent in mammalian membranes, Figure 1.1. This ergosterol binding affects membrane function and increases membrane permeability to protons and small ions (Georgopapadakou and Walsh 1994). The mechanism of action of polyenes is complex and also includes effects on the activity of fungal H^+-ATPase and results in fungicidal activity (Koller 1991). The complexity of the mechanism of action was demonstrated in a genomics study of the effect of amphotericin B on *Saccharomyces cerevisiae*, which found that the expression levels of 1455 genes was responsive to amphotericin B by at least 1.5 fold (Zhang et al. 2002).

The azole group of antifungal agents is the largest and most widely used, with 55 members used clinically in 2000 (Walsh et al. 2000) the first of which was discovered in the late 1970's (Alexander and Perfect 1997). Members of this group are highly lipophilic and fall into two main categories, imidazoles such as ketoconazole, and triazoles such as fluconazole, Table 1.1, with the older imidazoles being fungicidal and

the newer triazoles being fungistatic (Georgopapadakou and Walsh 1994). The mechanism of action of these compounds is complex and involves inhibition of two P450 enzymes involved in the biosynthesis of ergosterol, specifically $P450_{14\ DM}$ encoded by *ERG11* and Δ22-desaturase encoded by *ERG5* (Bossche et al. 2003), Figure 1.1. Inhibition of $P450_{14\ DM}$ occurs by formation of a complex between the azole ring of these antifungals and the heme iron (Georgopapadakou and Walsh 1996). Inhibition of $P450_{14\ DM}$ and Δ22-desaturase results in depletion of ergosterol and accumulation of 14-methylsterols. Since ergosterol is a vital component of fungal cell membranes responsible for membrane permeability and the activity of membrane bound enzymes, its depletion has major and complex consequences for fungal cells. For example, an mRNA profiling study of *Candida albicans* response to itraconazole found that 116 genes were at least 2.5 fold decreased, 180 were increased greater than 2.5 fold, and a global upregulation of genes involved in ergosterol biosynthesis was observed (De Backer et al. 2001).

The thiocarbamates and allylamines, although they are structurally very different, can be grouped together based on mechanism of action. Terbinafine, a member of the allylamines, and tolnaftate, a member of the thiocarbamates, Table 1.1, both noncompetitively inhibit fungal squalene epoxidase which is found in the cell membrane (Bossche et al. 2003), Figure 1.1. Squalene epoxidase is responsible for an early step in the biosynthesis of ergosterol in fungi (Ghannoum and Rice 1999) and cholesterol in higher eukaryotes (Favre and Ryder 1997). Inhibition of squalene epoxidase results in

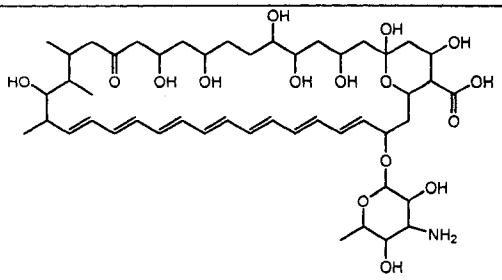
depletion of ergosterol and harmful buildup of squalene which alters membrane structure and function (Georgopapadakou and Walsh 1996; Ghannoum and Rice 1999).

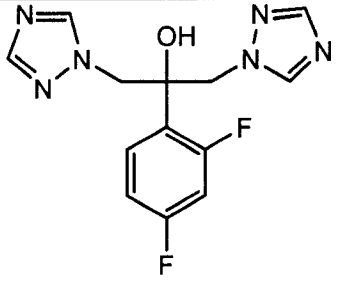
5-Fluorocytosine, Table 1.1, is a nucleoside analog that enters the cell using cytosine permease where it is deaminated into the well known anticancer drug, 5-fluorouracil by cytosine deaminase (Georgopapadakou and Walsh 1996). 5-Fluorocytosine was discovered in 1957 and found its first use in 1968 (Alexander and Perfect 1997). The selectivity of 5-fluorocytosine is due to little or no cytosine deaminase being present in mammalian cells whereas it is present in fungi (Bossche et al. 2003). The produced 5-fluorouracil can be further metabolized by the pyrimidine salvage pathway into 4-fluoro-uridine monophosphate, by the action of uridine monophosphate pyrophosphorylase, and then incorporated into RNA causing a disruption in protein synthesis (Bossche et al. 2003), Figure 1.1. The action of uracil phosphoribosyl transferase on 5-fluorouracil converts it into 5-fluoro-deoxyuridine monophosphate (Balkis et al. 2002), which inhibits thymidylate synthase, an enzyme that is essential to DNA synthesis (Bossche et al. 2003). 5-Fluorocytosine can be fungistatic or fungicidal depending on the drug concentration (Alexander and Perfect 1997), with the fungicidal activity likely due to interference with pyrimidine metabolism as well as RNA, DNA, and protein synthesis (Ghannoum and Rice 1999).

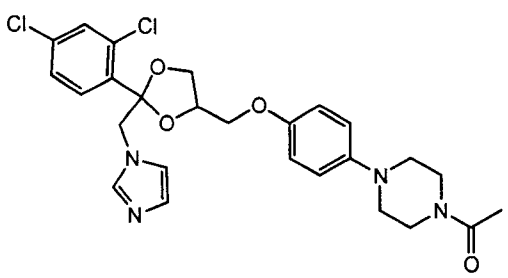
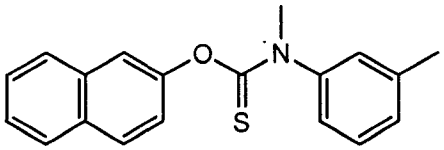
Caspofungin, Table 1.1, is a member of the echinocandins which are the most recently developed class of antifungals. The first members of the echinocandins were

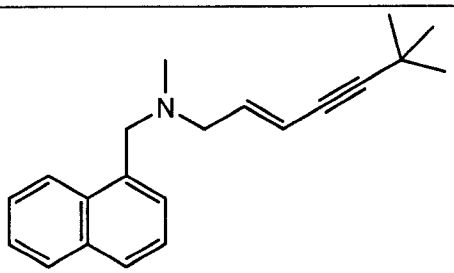
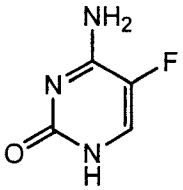
isolated in 1974 from an *Aspergillus* culture and chemical modifications in the 1980's led to compounds with better antifungal properties (Denning 1997). Caspofungin is produced by chemical modification of a compound from the fungus *Glarea lozoyensis* (Letscher-Bru and Herbrecht 2003), and was approved for clinical use in early 2002 (Denning 2002). These lipopeptide compounds form a novel class of antifungals because they non-competitively inhibit β-(1,3)-D-glucan synthase, a novel target among antifungal classes (Denning 1997), Figure 1.1, and are known to be fungicidal (Georgopapadakou and Walsh 1996). β-(1,3)-D-Glucan synthase is a heteromeric enzyme composed of a catalytic enzyme, Fks1, and a regulatory subunit, Rho1p, located on the cell membrane (Letscher-Bru and Herbrecht 2003). The complex polymerizes uridine diphosphate-glucose into β-(1,3)-D-glucan which is extruded out of the cell where it is incorporated into the cell wall giving the cell shape and mechanical strength (Letscher-Bru and Herbrecht 2003). The spectrum of antifungal activity against pathogenic fungi is not complete, owing to the lack of β-(1,3)-D-glucan in some fungal cell walls and difficulties penetrating to the target in some fungi (Letscher-Bru and Herbrecht 2003). Since mammalian cells do not use β-(1,3)-D-glucan the compound is selective against fungal cells and produces few side effects in mammals.

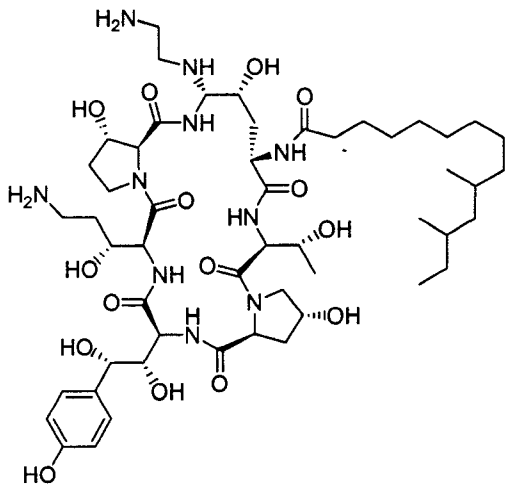
Table 1.1. Clinically important antifungals.

Compound Name, Class	Structure, Discovery, Mechanism of Action, and Target Information	Resistance
Amphotericin B, Polyene	 - introduced in the 1950s - binds ergosterol in fungal cell membrane causing increased permeability - fungicidal	- reduced ergosterol content of the cell membrane and replacement of ergosterol with other sterols which have decreased affinity for the polyenes

Fluconazole, Azole (Triazole)	 - introduced in early 1990s - complex mechanism of action, although primarily inhibits $P450_{14\ DM}$ in the ergosterol biosynthetic pathway leading to depletion of ergosterol and buildup of toxic intermediates - fungistatic	- decreased drug influx - increased drug efflux using ABC or MFS type efflux pumps - amino acid substitutions in $P450_{14\ DM}$ that reduce binding - gene amplification of *ERG11* and also genes of efflux pumps

Ketoconazole, Azole (Imidazole)	 - introduced in late 1980s - complex mechanism of action, inhibits $P450_{14\ DM}$ and Δ22-desaturase in the ergosterol biosynthetic pathway, effect same as fluconazole - fungicidal	- same as for fluconazole
Tolnaftate, Thiocarbamate	 - inhibits squalene epoxidase resulting in depletion of ergosterol and accumulation of toxic sterols in the membrane	- active efflux using ABC type efflux pumps

Terbinafine, Allylamines	 - inhibits squalene epoxidase resulting in depletion of ergosterol and accumulation of toxic sterols in the membrane	- active efflux using ABC type efflux pumps
5-Fluorocytosine, Nucleoside Analog	 - discovered in 1957, introduced in 1968 - taken up by fungal cells, phosphorylated and incorporated into RNA causing miscoding - also further converted into deoxynucleoside that inhibits thymidylate synthase inhibiting DNA synthesis - usually used in combination to avoid facile selection of resistance - fungistatic or fungicidal depending on drug concentration	- loss of cytosine permease activity decreases uptake - changes in cytosine deaminase or uridine monophosphate pyrophosphorylase results in accumulation of harmless 5-fluorocytosine

Caspofungin, Echinocandin	 - developed in the 1980's, introduced in 2002 - inhibits β-(1,3)-D-glucan synthase which makes β-(1,3)-D-glucan found in the cell wall - fungicidal	- clinical failure yet to be observed - some clinical strains have been shown to have decreased MIC due to the activity of *CDR2* ABC type efflux pump

1.3. Mechanisms of Antifungal Resistance

Despite the fact that the polyene antifungals have been in use for 50 years, there is little acquired resistance to them. There are a number of fungal species that have primary resistance to polyene antifungals, in that all strains of these species are uneffected by the polyenes (Masia Canuto and Gutierrez Rodero 2002). Such primary resistance is common among emerging pathogenic fungi (Georgopapadakou and Walsh 1996), which do not contain sterols in their outer membrane (Ghannoum and Rice 1999). What little acquired or secondary resistance that is found is generally confined to specific and less

common species of *Candida* (Balkis et al. 2002). In these species, polyene resistance occurs by reductions in the ergosterol content of fungal cell membranes or replacement of ergosterol with other sterols which have a lower affinity for the polyenes (Ellis 2002).

Resistance to azole antifungal agents was minimal prior to 1986. Since then, fungi have acquired the ability to prevent toxic accumulations of azoles and have evolved an altered target, $P450_{14\,DM}$. Strains have been found which have decreased drug influx (Alexander and Perfect 1997) and others with increased drug efflux using either ATP binding cassette (ABC) efflux pumps, including the *Candida* drug resistance pumps (CDR) *CDR1* and *CDR2*, or major facilitator superfamily (MFS) pumps (Ghannoum and Rice 1999). ABC transporters can transport antifungal agents and other solutes against a concentration gradient by harnessing the energy in ATP (Del Sorbo et al. 2000). MFS pumps accomplish the same feat using proton-motive force composed of the membrane potential and electrochemical proton gradient (Del Sorbo et al. 2000). Many amino acid changes have been found in $P450_{14\,DM}$ which generally reduce drug binding with some also reducing catalytic activity of the enzyme (Loeffler and Stevens 2003). Lastly, gene amplification is used by fungi to increase the number of *ERG11* genes and thereby increase the amount of enzyme present, however, this form of resistance is likely the least important (Ghannoum and Rice 1999). Such a strategy has also been found for increasing the number of efflux pumps (Ghannoum and Rice 1999). In general, pathogenic strains that resist azole antifungals use a variety of these mechanisms at one time to overcome the drug (White 1997; Masia Canuto and Gutierrez Rodero 2002).

Little is known about resistance mechanisms to thiocarbamates and allylamines as little resistance has been encountered (Koller 1991). However, there have recently been reports of resistance to these fungicides by active efflux using the *CDR1* and *CDR2* efflux pumps (Balkis et al. 2002; Bossche et al. 2003).

Fungal resistance to nucleoside analogs develops very quickly obviating the use of 5-fluorocytosine in monotherapy (Bossche et al. 2003). However, resistance can be avoided by using 5-fluorocytosine in combination with other antifungal agents. Since many enzymes are needed for the fungicidal activity of 5-fluorocytosine and there are multiple targets, fungi have numerous ways of developing resistance to the nucleoside analog (Alexander and Perfect 1997). For example, loss of permease activity will decrease uptake, or changes in either cytosine deaminase or uridine monophosphate pyrophosphorylase will result in accumulation of the harmless 5-fluorocytosine inside the cells and no conversion of the prodrug into the active form (Ghannoum and Rice 1999).

Caspofungin has been clinically used for just over a year and early reports showed a lack of acquisition of clinical resistance (Balkis et al. 2002; Letscher-Bru and Herbrecht 2003). However, resistance can be selected for in the lab by mutations in either Fks1 or Rho1p (Kurtz et al. 1996; Douglas et al. 1997; Letscher-Bru and Herbrecht 2003). Furthermore, the Golgi protein Sbe2p, encoded by *SBE2*, when overexpressed was found to provide resistance to caspofungin by an unknown mechanism (Osherov et al. 2002). Since Sbe2p is involved in cell wall formation, this finding may be a prelude to a possible

resistance mechanism and may explain why some fungi are intrinsically resistant to caspofungin (Osherov et al. 2002). Lastly, a recent report implicates *CDR2* and to a much lesser extent, *CDR1*, in increased minimum inhibitory concentrations of caspofungin when tested on agar plates but not when assessed using the NCCLS microdilution method (Schuetzer-Muehlbauer et al. 2003).

From these specific descriptions of antifungal resistance, one can see a troubling picture that fungi have or are getting the upper hand in this ongoing battle. A comparison to bacterial antibiotic resistance is useful to put things in perspective. There are far more antibiotics in clinical use compared to antifungal agents and the number of cellular targets is much greater than the four targeted by clinical antifungals. However, bacteria have a very large number of resistance mechanisms and the ability to transfer these mechanisms between different species. Thankfully, fungi do not have as many resistance mechanisms and do not have the ability to transfer these mechanisms between species (Ghannoum and Rice 1999; Hof 2001). Therefore, the antifungal resistance picture is not as bleak as one might believe from reading a summary of antifungal resistance mechanisms. This, however, should not encourage us to believe that we are far ahead in the battle. To the contrary, we must solidify our advantage with new agents against new targets to ensure that cross resistance between current and new antifungals does not develop.

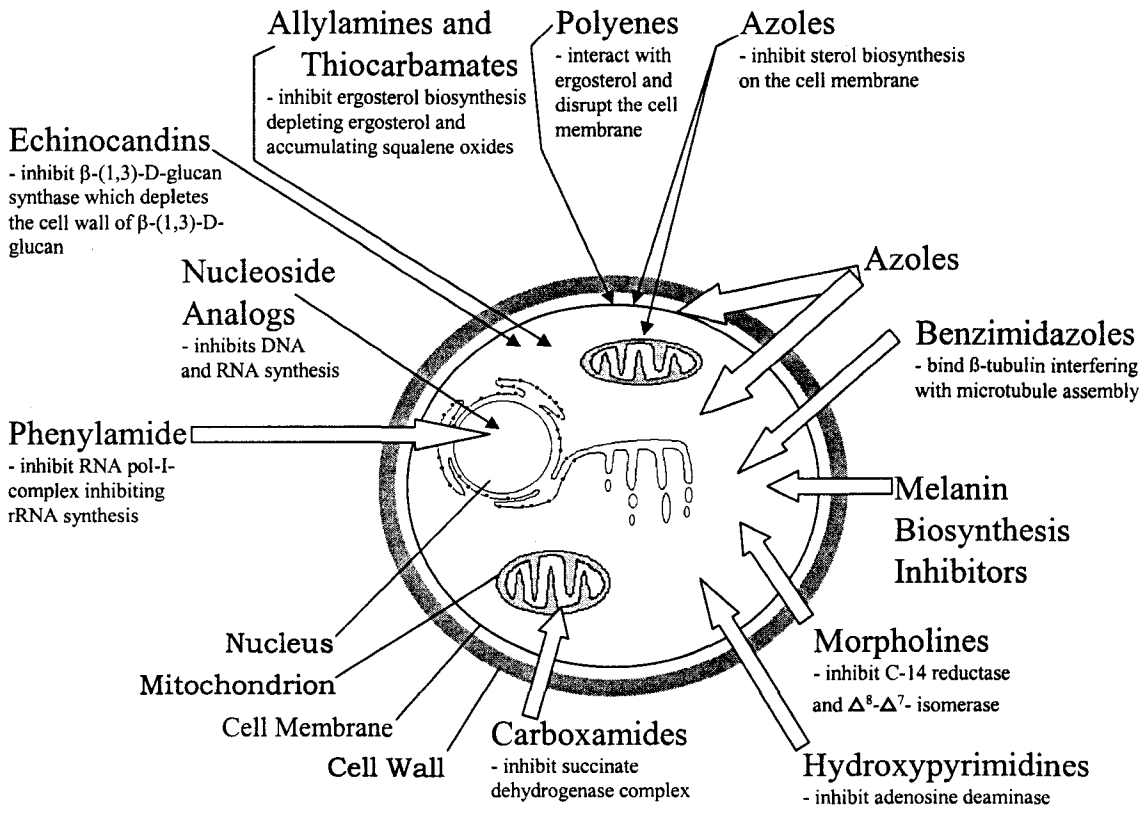


Figure 1.1. Fungal Targets of antifungal compounds (narrow arrows) and fungicides (wide arrows).

1.4. Fungicides and Resistance to Fungicides

A subset of antifungals used in humans are approved for use in animals along with other compounds restricted to use in agriculture. However, it should be noted that fungal infections of animals are a very significant source of infections in humans. For example, up to 80% of human skin fungal infections may be of animal origin and, in urban environments, up to 20% of such infections can be traced back to dogs or cats (Rochette et al. 2003). Those compounds used in agriculture are commonly called fungicides, although many of the compounds do not possess cidal activity, (Hof 2001). The use of the term fungicide, in this section, will refer to compounds with general antifungal activity which may or may not be cidal.

Fungicides have been in use for over 200 years, with early uses including the "Bordeaux mixture" of lime and copper sulfate which was used to prevent downy mildew fungus on grapes in 1882 (Hudler 1998). Currently, over 135 chemicals are used as fungicides globally (Muir and White 1999). Studies have shown that use of fungicides gives farmers a cost : benefit ratio of 1 : 3 (Muir and White 1999). Resistance to fungicides is a large problem, not only for economic reasons, but also because it can spread very quickly over large geographic areas as fungal spores can travel great distances on the wind (Hudler 1998). Bacterial resistance is frequently encoded on mobile elements of DNA, such as transposons, insertion sequences, bacteriophages and plasmids, which allows the spread of resistance among different species inhabiting the

same environment. On the other hand, fungi have not been found to pass resistance genes from one species to another, thus resistance tends to remain in specific fungal species (Ghannoum and Rice 1999; Hof 2001). A last item of concern is the possible selection of antifungal resistance in fungi in the agricultural sector which then become problematic in human medicine. An excellent example is the heavy use of azole fungicides on crops which could select for resistance in fungi found in the field that are also human pathogens. While this has never been documented, it does not obviate the possibility (Hof 2001). Similarly, fungicide residues found on foods ingested by humans may provide selective pressure in humans for antifungal resistance (Hof 2001).

Phenylamides, Table 1.2, inhibit the RNA polymerase I-template complex thereby inhibiting rRNA synthesis, Figure 1.1 (Koller 1991). Resistance to these compounds occurs by amino acid changes in RNA polymerase that decrease fungicide binding (Brent 1995), and was found within 2 years of their first use in 1977 (Muir and White 1999).

Carboxamides, including flutolanil, Table 1.2, are old fungicides that inhibit succinate dehydrogenase, Figure 1.1 (Steffens et al. 1996). Resistance to carboxamides is low, but an amino acid change in succinate dehydrogenase has been found to confer resistance (Steffens et al. 1996). These compounds enjoyed 15 years without resistance from their time of introduction in the early 1970s (Brent 1995).

Hydroxypyrimidines, such as bupirimate, Table 1.2, work by inhibiting adenosine deaminase in the purine salvage pathway, Figure 1.1 (Koller 1991). Inhibition of the purine salvage pathway is effective in fungi that are purine auxotrophs where salvage of host purines is required for fungal survival (Koller 1991). Resistance to hydroxypyrimidines was identified soon after their commercial use (Koller 1991).

Benzimidazoles, including Benomoyl, Table 1.2, are fungicides that bind to the fungal β-tubulin protein thereby preventing the non-covalent polymerization of α,β-tubulin into stable microtubules, Table 1.1 (Steffens et al. 1996). Resistance, now a major problem, was found in the field within two years of introduction in the 1970's (Muir and White 1999), and is caused by point mutations (Hollomon et al. 1998). Interestingly, these amino acid changes cause increased sensitivity to phenylcarbamates which allows mixtures of fungicides to be used to combat fungi and delay or overcome resistance.

Melanin biosynthesis inhibitors, such as tricyclazole, Table 1.2, inhibit the polyketide pathway of fungal melanin biosynthesis resulting in abnormal accumulation of melanin precursors, Figure 1.1 (Koller 1991). The process of fungal penetration of plant tissue is complex and can occur by several mechanisms. One such mechanism used by the rice blast fungus, *Magnaporthe grisea*, initiates when a spore lands on a plant surface, sporulates, and exudes a glue-like substance at one end which eventually tightly attaches the spore to the plant surface (Howard and Valent 1996). A new cell wall layer of

melanin is deposited just outside the plasma membrane on the entire surface except where the cell is in contact with the plant surface, which is called the appressorium pore (Howard and Valent 1996). At this point, perhaps the largest turgor pressures found in nature are applied inside the fungal cell and are held by the melanin coated cell wall and directed through the region without melanin to the plant surface somehow being transformed, with other factors, into mechanical force which breaks into the plant cell (Howard and Valent 1996). Melanin defective mutants have been shown to be unable to infect intact plant surfaces but remain viable (Howard and Valent 1996), thus melanin biosynthesis inhibitors can only be used to prevent fungal infection or prevent further spread of an existing infection to other plants (Koller 1991).

Azole group of fungicides, Table 1.2, have been in use since 1970 (Brent 1995) and are sometimes referred to as demethylation inhibitors because they act against $P450_{14\,DM}$ in the ergosterol biosynthetic pathway, Figure 1.1 (Steffens et al. 1996). The 35 separate compounds belonging to this group have found use in agriculture against phytopathogenic fungi (Brent 1995). They have two major advantages over other fungicides. First, they are very stable in certain ecological niches, for example the half life in the soil can be greater than 100 days, and second, they can be used to treat plants that have established fungal infections rather than being used only as a preventative as many other fungicides are (Hof 2001). Although amino acid changes in $P450_{14\,DM}$ have been found in human pathogenic fungi treated with azoles, similar resistances have yet to be identified in the field (Steffens et al. 1996). Active efflux mechanisms have been

found in the field (Steffens et al. 1996) although such incidences have been sporadic and with fluctuating severity (Brent 1995).

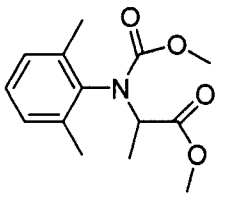
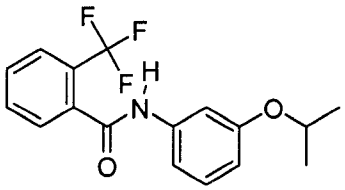
Morpholines, including tridemorph, Table 1.2, act by inhibiting the C-14 reductase and Δ^8-Δ^7- isomerase enzymes in the ergosterol biosynthetic pathway, Figure 1.1 (Brent 1995). Resistance to this group of fungicides has been remarkably small considering the ease at which resistance can be selected for in the lab (Brent 1995). The fact that these compounds inhibit more that one enzyme may be the reason why resistance in the field is not widely found (Brent 1995).

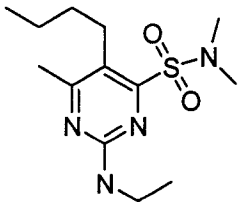
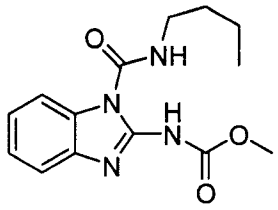
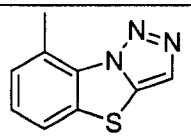
Strobilurin fungicides, Table 1.2, were discovered in the late 1970's but weren't developed until the early 1990's. Sales began in 1996 and grew to 10% of the global fungicide market with current sales totalling 600 million dollars U.S. (Bartlett et al. 2002). These compounds are fungicidal and work by inhibiting mitochondrial respiration by inhibiting Cytochrome b on the inner mitochondrial membrane, Figure 1.1 (Bartlett et al. 2002). Resistance to these compounds has been found in the field, but its incidence remains regional. Resistance occurs by amino acid substitutions in Cytochrome b, specifically G143A and F129L (Bartlett et al. 2002).

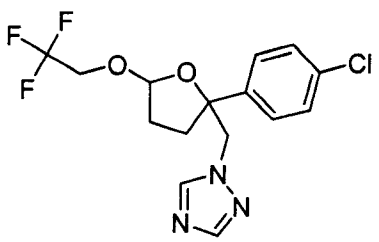
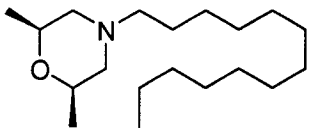
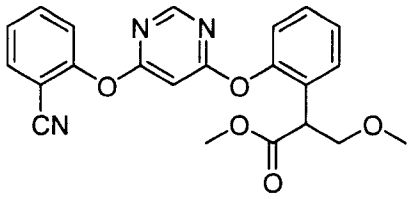
Dicarboximides, including vinclozolin, Table 1.2, are important fungicides that have encountered low level resistance in the field by an unknown mechanism. The compounds are known to inhibit a serine/threonine protein kinase, but how this translates

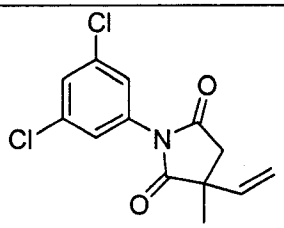
into fungicidal activity is unknown (Steffens et al. 1996). These compounds were first used in the late 1970s and went 5 years before the first resistance was reported (Muir and White 1999).

Table 1.2. Agriculturally important fungicides.

Compound Name, Class	Structure, Discovery, Mechanism of Action, and Target Information	Resistance
Metalaxyl, Phenylamides	 - used initially in Holland in 1980 - targets RNA polymerase I – template complex	- resistance arises easily by amino acid changes at the binding site
Flutolanil, Carboxamides	 - initially discovered in 1930's but useful derivatives were not found until 1966 - inhibit the succinate dehydrogenase complex	

Bupirimate, Hydroxypyrimidines	 - discovered in 1960's - inhibit adenosine deaminase of the purine salvage pathway - many fungi use host derived purines either partially or totally	
Benomyl, Benzimidazoles	 - discovered in the late 1960's - bind β-tubulin interfering with microtubule assembly	- resistance is widespread - single amino acid changes reduce binding
Tricyclazole, Melanin Biosynthesis Inhibitors	 - inhibits melanin biosynthesis which is needed for fungal penetration of plant cells - not fungicidal, acts as a preventative	

Furconazole, Azoles	 - developed in the late 1960's - targets $P450_{14\ DM}$	
Tridemorph, Morpholines	 - developed in the late 1960's - targets sterol C-14 reductase and sterol Δ^8-Δ^7- isomerase	- found in lab strains but not in the field
Azoxystrobin, Strobilurins	 - discovered in the late 1970's but developed in early 1990's - inhibits Cytochrome b	- limited occurrences in the field due to amino acid changes in Cytochrome b

Vinclozolin, Dicarboximides	 - inhibits a serine/threonine protein kinase	- often find low level resistance in the field but mechanism is unknown

1.5. The Aspartate Pathway: A New Antifungal Target

The battle against pathogenic microorganisms is one that is ongoing. In prokaryotes, the battle is aided by their unique physiology and structures. Unfortunately, there are few differences between fungi and mammals (Hector 1993), so the ability to selectively inhibit growth of fungi as opposed to mammalian cells is challenging, but not impossible. For example, cell wall differences are aptly taken advantage of by the candin antifungals, while specific metabolic differences, such as the ergosterol biosynthetic pathway and cytosine deaminase are targeted by allylamines, thiocarbamates, azoles, and 5-fluorocytosine respectively. Regrettably, these differences do not encompass all fungi, so the spectrum of activity of many of the clinically used antifungal agents is limited. For example, ergosterol is present in most but not all fungi (Ghannoum and Rice 1999). When viewed from this, it is obvious that new targets should be sought and that such targets should include all fungi so that antifungals with a broad spectrum of activity can be developed.

The fungal aspartate pathway, Scheme 1.1, for the biosynthesis of L-Thr, L-Met, and L-Ile appears to fulfill these requirements as all fungi require these amino acids and all have the biosynthetic pathway. However, mammals must obtain these amino acids in their diet as they do not possess an equivalent biosynthetic pathway. Plants and bacteria, on the other hand, possess a slightly different aspartate pathway compared to the fungal version. In plants and bacteria, the aspartate pathway has an additional branch from aspartate-4-semialdehyde that leads to the biosynthesis of L-Lys (Truffa-Bachi et al. 1974). This branch is important not only for the biosynthesis of L-Lys, but the last intermediate prior to L-Lys, diaminopimelate, is a major precursor for bacterial cell wall biosynthesis (Cummins and Harris 1956; Galili 2002). The importance of this branch is underscored by the fact that bacteria possess three independent pathways that lead to diaminopimelate, with some bacteria using two of these pathways (Schrumpf et al. 1991; Wehrmann et al. 1998). Another significant difference between the fungal pathway and the bacterial pathway is the presence of three aspartate kinase activities in bacteria, with two of these being bifunctional enzymes with homoserine dehydrogenase, that are allosterically regulated by three different pathway products. For example, *Escherichia coli* aspartate kinase I – homoserine dehydrogenase I responds to L-Thr and L-Ile, aspartate kinase II – homoserine dehydrogenase II responds to L-Met, and aspartate kinase III responds to L-Lys.

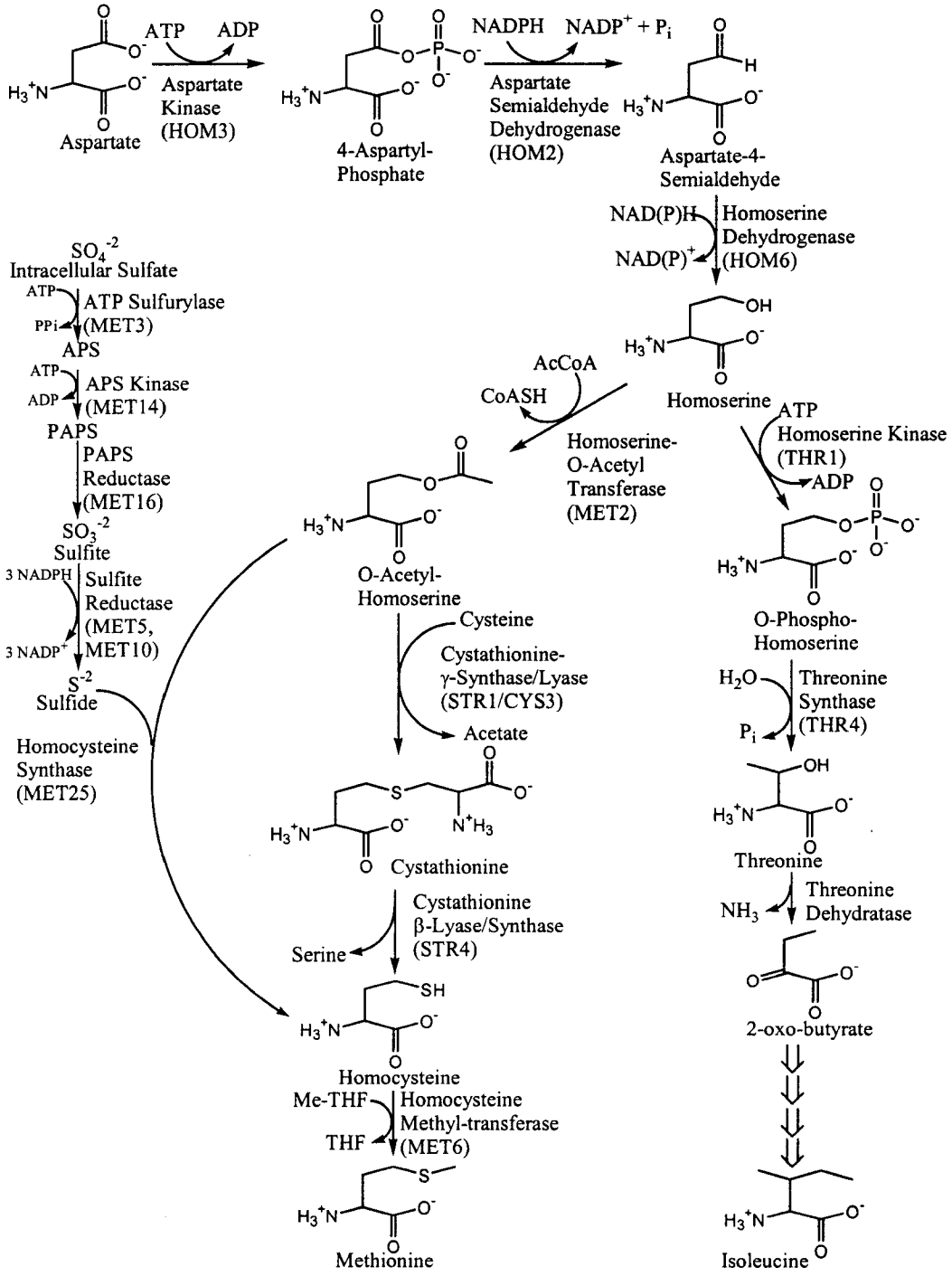
The products of the aspartate pathway are very important to organisms which make them. Obviously the amino acids made by the pathway are essential for protein

synthesis, particularly methionine, universally the first amino acid in all proteins. As previously mentioned diaminopimelate is an essential component in bacterial cell walls. Methionine made by the pathway is used to make *S*-adenosylmethionine (SAM) which is used in a vast assortment of cellular processes involving methylation. An important use of SAM in bacteria, including some pathogens, is in the LuxI protein family directed synthesis of acyl-homoserine lactones which are used for quorum sensing to avoid starvation caused by unsustainable cell densities as well as controlling virulence gene expression (Huisman and Kolter 1994; Parsek et al. 1999). The fact that mutants with defective quorum sensing systems show reduced virulence, has prompted the idea that quorum sensing could be a target for new antibiotics by blocking production or reception of the acyl-homoserine lactone signal (Finch et al. 1998). Since the acyl-homoserine lactone synthases use SAM in a mechanism that differs from the mechanism of all other SAM utilizing enzymes, molecules which target these synthases would likely be specific (Parsek et al. 1999). An inviting idea is the possibility that inhibitors to quorum sensing systems may be species specific, allowing treatments specific for pathogens while the natural flora is uneffected (Strauss 1999). In plants, SAM is the precursor to 1-aminocyclopropane-1-carboxylic acid, which in turn leads to ethylene production (Ravanel et al. 1998), a major signal for fruit ripening (Alexander and Grierson 2002).

The aspartate pathway is also very important to human nutrition because half of the amino acids that humans must obtain from their diet come from the aspartate pathway (Hesse et al. 2001). The vast majority of these amino acids in humans' diet come from

plant material, however, in many plants the abundance of these amino acids is not optimal (Galili and Hofgen 2002). For this reason, much effort has been, and continues to be, focused on increasing the amino acid content of plants, in the past, by traditional plant breeding, but now, including the use of biotechnology (Brinch-Pedersen et al. 1996; Wang et al. 2001; Zeh et al. 2001).

It has been previously suggested that amino acid biosynthetic pathways would not make a good target as fungi are likely capable of circumventing the pathway by obtaining the pathway products from the host (Koller 1991). For example, none of the aspartate pathway enzymes were found to be essential in *Bacillus subtilis* when gene inactivations were isolated on rich media (Kobayashi et al. 2003), indicating *Bacillus* has no difficulty acquiring these amino acids from the media. Two arguments can used against this thought. First, the concentration of L-Thr, L-Met, and L-Ile may not be sufficiently high in the infection zone to allow fungal amino acid transporters to satisfy the requirements of the fungi. Concentrations of L-Thr, L-Met, and L-Ile in the blood of mammals such as humans, rats, and cows range from 90 - 420, 22 – 120, and 72 – 370 μM, respectively (Fafournoux et al. 1990; Pacheco-Rios et al. 1999; Tietge et al. 2002). Secondly, there are several known natural products, Figure 1.2, which inhibit the aspartate pathway and have been shown to prevent infection in animal models.



Scheme 1.1. Aspartate pathway for fungal biosynthesis of L-Thr, L-Ile, L-Met.

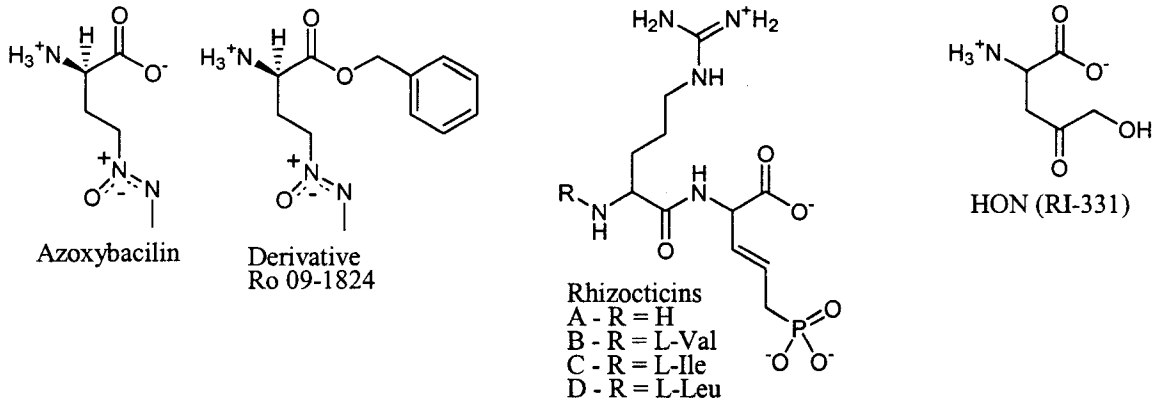


Figure 1.2. Structures of Azoxybacilin and a derivative Ro 09-1824, Rhizocticin A, B, C, D, and (S)-2-amino-5-hydroxy-4-oxopentanoic acid (HON) also known as RI-331.

Azoxybacilin, Figure 1.2, is a secondary metabolite isolated from *Bacillus cereus* Frankland found during a screening program for novel antifungal agents of microbial origin (Fujiu et al. 1994). The compound was found to inhibit fungal growth by inhibiting methionine biosynthesis at the sulfur fixation step. The only tolerated structural change was esterification of the carboxyl with a benzyl ester (Ro 09-1824) which resulted in an IC_{80} of 2.1 μg/mL (Ohwada et al. 1994). The mode of action of azoxybacilin was determined to be inhibition of a step or steps in the sulfur-fixation pathway, which represents the first inhibitor of such activity (Aoki et al. 1994). A further study by the same group found that azoxybacilin interferes with the regulation of transcription of *MET4* which represses the transcription of the five genes involved in sulfate assimilation: *MET3* (ATP sulfurylase), *MET14* (APS kinase), *MET16* (PAPS reductase), *MET10* (sulfite reductase), and *MET25* (homocysteine synthase), with *MET10*

induction being inhibited 10 times more than the other genes (Aoki et al. 1996). Inhibition of the enzymes involved in sulfate assimilation was not found (Aoki et al. 1996).

The rhizocticins, Figure 1.2, are di or tripeptides produced by *Bacillus subtilis* ATCC 6633 containing the unusual L-2-amino-5-phosphono-3-*cis*-pentenoic acid (L-APPA). It is assumed that the tripeptide nature allows transport into fungal cells where peptidases liberate L-APPA which is assumed to inhibit threonine biosynthesis (Kugler et al. 1990). L-APPA was later shown to inhibit *E. coli* threonine synthase, with a K_i of 100 μM (Laber et al. 1994). The plumbemycins are related tripeptides due to the presence of L-APPA, but the other two amino acids direct L-APPA into bacterial rather than fungal cells (Fredenhagen et al. 1995).

The amino acid analog, (S)-2-amino-5-hydroxy-4-oxopentanoic acid (HON) is also known as RI-331, Figure 1.2. HON, originally isolated in a screen for antituberculous antibiotics (Kanazawa et al. 1959; Tatsuoka et al. 1960), is produced by *Streptomyces* sp. and has been shown to inhibit a variety of fungi, be well tolerated by mice and protect mice from a pathogenic strain of *Candida albicans* (Yamaguchi et al. 1988). HON also inhibits the growth of some, but not all, plant pathogens (Yamaki et al. 1992b). The compound was found to hinder protein synthesis by inhibiting the biosynthesis of threonine, methionine, isoleucine and serine (Yamaki et al. 1988). HON acts in a fungistatic rather than fungicidal manner as it prevents the growth of, but does

not kill, *Saccharomyces* (Yamaguchi et al. 1990). In the same paper, HON was found to dramatically decrease the pools of threonine, methionine and isoleucine and consequently the synthesis of protein was found to be greatly diminished. Exogenous addition of these amino acids, especially homoserine, was shown to overcome the antifungal activity of HON (Yamaguchi et al. 1990). Assays of crude aspartate kinase, aspartate semialdehyde dehydrogenase, and homoserine dehydrogenase showed that HON only inhibits homoserine dehydrogenase and acts as a mixed inhibitor versus aspartate semialdehyde with a K_i of 8 mM (Yamaki et al. 1990). Further investigations of the kinetics of homoserine dehydrogenase inhibition by HON revealed that greater inhibition occurs in the forward direction when the product $NADP^+$ is added, giving a K_i of 2 mM and mixed inhibition vs aspartate semialdehyde (Yamaki et al. 1992a). The inhibition was much greater in the reverse direction, with HON acting as a competitive inhibitor versus homoserine with a K_i of 25 μM (Yamaki et al. 1992a). Studies of the biosynthesis of HON in *Streptomyces akiyoshiensis* have selected mutants incapable of making HON (Le et al. 1996) and the relation between culture media and production of HON has been studied (Glazebrook et al. 1993). Supplying growing *Streptomyces* with radiolabelled substrates showed four carbons are derived from a citric acid cycle compound and the methylene carbon comes from acetate. Another study showed that the four carbons could be supplied by L-Asp (White et al. 1994). HON has also been shown to inhibit δ-aminolevulinic acid dehydratase from *Mycobacterium phlei* in a competitive manner,

which is not unexpected as HON is similar in structure to the substrate δ-aminolevulinic acid (Yamasaki and Moriyama 1971).

1.6. The Enzymes of the Aspartate Pathway

1.6.1. Aspartate Kinase

Aspartate kinase (AK) is the first enzyme in the aspartate pathway, Scheme 1.1, and is responsible for the phosphorylation of aspartate to the unstable compound 4-phosphoaspartate. The single *S. cerevisiae* AK (AK_{Sc}) is regulated by L-Thr inhibition (Stadtman et al. 1961), with 3 mM L-Thr reducing the activity approximately 50% (Ramos et al. 1991). The yeast *HOM3* gene was first cloned by complementation of a *HOM3* mutant, (Rafalski and Falco 1988), however, due to a sequencing error which changed the reading frame, the stop codon was identified incorrectly, but was later corrected (Rafalski and Falco 1990). The *HOM3* gene codes for 527 amino acids which gives a molecular weight of 58 kD.

The biochemistry of the *E. coli* AKs has been studied in much greater detail than AK_{Sc}. The kinetic mechanism of $AKIII_{Ec}$ was shown to be random binding of L-Asp and ATP followed by ordered release of ADP and subsequently 4-phosphoaspartate (Shaw and Smith 1977). More recently, the kinetic mechanism of the AK portion of AKI-$HSDI_{Ec}$ was studied and found to be identical to the previous study of $AKIII_{Ec}$ (Angeles

and Viola 1990). Chemical modification and pH studies of AKI-$HSDI_{Ec}$ have implicated several histidines and tyrosines as being important for structure and activity (Angeles et al. 1989). Similar studies with $AKIII_{Ec}$ have shown cysteine and histidine are important for activity (Keng and Viola 1996). AKI_{Ec} has been shown to have the surprising ability to phosphorylate β-derivatized substrates, indicating that the enzyme can phosphorylate the α-carboxyl group when the β-carboxyl group is derivatized in a manner that prevents it being a phosphate acceptor (Angeles et al. 1992). Similar observations were made with $AKIII_{Ec}$ (Keng and Viola 1996).

Studies of the allosteric regulation of AK_{Sc} have shown several amino acids are important to the process. A chemically induced mutant of *HOM3* could not be grown in the presence of L-Thr and was shown to have a mutation causing the amino acid substitution AK_{Sc}-A406T which increases the L-Thr inhibition by 30 fold (Arevalo-Rodriguez et al. 1999). Another mutant of *HOM3* gave an amino acid substitution, AK_{Sc}-D452G, that resulted in AK_{Sc} being insensitive to L-Thr inhibition and allowing *Saccharomyces* expressing this enzyme to accumulate 8 fold more L-Thr (Martin-Rendon et al. 1993). Other amino acid substitutions within AK_{Sc} have been shown to have varying increases in sensitivity to L-Thr inhibition (Arevalo-Rodriguez et al. 1999).

An interesting observation about the aspartate pathway in *E. coli* is the fusion of the first and third catalytic activities in the bifunctional enzymes AKI-$HSDI_{Ec}$ and AKII-$HSDII_{Ec}$, where one would have expected fusions between consecutive reactions. A

partial explanation for this was recently established by the observation that ASD_{Ec} forms a protein-protein complex with the bifunctional AKI-$HSDI_{Ec}$, and also with the monofunctional $AKIII_{Ec}$, expressly to allow direct transfer of 4-phosphoaspartate from the AK domain to ASD_{Ec} (James and Viola 2002). This makes excellent biological sense because 4-phosphoaspartate is unstable and the expensive use of ATP to activate aspartate would be wasted if 4-phosphoaspartate were to freely diffuse within the cell where a substantial amount would degrade back to aspartate. The portion of the puzzle yet to be shown, is whether the product of aspartate semialdehyde dehydrogenase, aspartate-4-semialdehyde, is channeled to homoserine dehydrogenase or whether it is allowed to freely diffuse within the cell.

The vast sequencing efforts of the late 1990's and subsequent bioinformatic analysis of the generated sequences is very useful in identifying conserved domains within families of proteins. On such domain was identified in AKs and is called the ACT domain, named for three of the allosterically regulated enzymes which contain the domain, aspartate kinase, chorismate mutase, and TyrA (prephenate dehydrogenase) (Aravind and Koonin 1999). These domains are frequently found in a tandem repeat of two ACT domains of similar sequence (Chipman and Shaanan 2001). Recently a new domain, called the RAM domain for Regulation of Amino acid Metabolism, was identified and shown not to be similar to the ACT domain at the DNA level, however, similarity is observed at the protein level as both domains are composed of a βαββαβ-fold (Ettema et al. 2002).

Similarities between regions of AK and other proteins have been used to suggest relationships with AKs. One example was the comparison of 45 amino acids from UMP-kinase with a region in AKs. The observed similarity was taken as evidence that UMP-kinase belongs in the AK family of kinases (Serina et al. 1995). More recently, a cataloging of all known kinase sequences into groups and families revealed that AKs make up a family of enzymes which include carbamate kinase, acetylglutamate kinase, glutamate 5-kinase, and uridylate kinase and fall in the Rossmann-like group of kinases (Cheek et al. 2002). A crystal structure of carbamate kinase is known (Ramon-Maiques et al. 2000) and the mechanism of phosphoryl transfer has been investigated (Ramon-Maiques et al. 2002). In the aforementioned listing of kinases, HSKs appear in 2 families that are in separate groups (Cheek et al. 2002). The second HSK from *Pseudomonas aeruginosa*, which can complement an *E. coli* HSK mutant as well as a phosphoserine phosphatase mutant (Patte et al. 1999), belongs in the L-2-haloacid dehalogenase family which also falls in the Rossmann-like group (Cheek et al. 2002). Studies of phosphoserine phosphatase show that the γ-phosphate is transferred to an enzyme aspartate residue in a conserved motif during catalysis (Collet et al. 1998), this motif is not found in AKs.

1.6.2. Aspartate Semialdehyde Dehydrogenase

Aspartate semialdehyde dehydrogenase (ASD) is responsible for the reduction of 4-phosphoaspartate to aspartate-4-semialdehyde exclusively using NADPH as a cofactor, Scheme 1.1. The enzyme from *S. cerevisiae* (ASD_{Sc}) was purified and found to be a tetramer (Holland and Westhead 1973). The first plant ASD was recently cloned, overexpressed, and purified from *Arabidopsis thaliana* (Paris et al. 2002). The *E. coli* ASD (ASD_{Ec}) was found to be a dimer with a subunit molecular weight of 38 kD, which transfers the *pro*-S hydrogen of NADPH to 4-phosphoaspartate, and one cysteine was suggested to be important for activity (Biellmann et al. 1980). Kinetic studies on the ASD_{Ec} revealed that the enzyme binds NADPH followed by 4-phosphoaspartate in a preferred random order and has an ordered release of products: aspartate semialdehyde, $NADP^+$, followed by inorganic phosphate (Karsten and Viola 1991). Furthermore, pH titrations and chemical modification studies were consistent with a cysteine and lysine residue being important for chemistry (Karsten and Viola 1991). The cysteine was later identified as the active site nucleophile by mutating the gene to give ASD_{Ec}-C135A and ASD_{Ec}-C135S which retained 0 and 0.3% of the wild type activity (Karsten and Viola 1992). The *HOM2* gene codes for ASD in *S. cerevisiae* and the sequence of the gene was found to code for 365 amino acids giving a molecular weight of 39.5 kD (Thomas and Surdin-Kerjan 1989).

The crystal structure of the apo-enzyme of ASD_{Ec} has been determined, allowing the active site to be identified along with a proposed catalytic base, H274 (Hadfield et al. 1999). More recently, the crystal structure of ASD_{Ec} with NADPH as well as with a substrate analog that covalently modifies the active site nucleophile, showed that binding of the cofactor induces a conformational change creating the binding site for 4-phosphoaspartate (Hadfield et al. 2001). This differs with ASD from *Vibrio cholerae* which was found to have similar active site structures with and without substrates (Blanco et al. 2003).

The product of the ASD reaction sits at the first branch point in the plant and bacterial aspartate pathways, one side leads to biosynthesis of L-Lys and the other to L-Thr, L-Ile, and L-Met. As previously mentioned, the pathway branch leading to L-Lys has an additional importance, as it supplies diaminopimelate for cell wall biosynthesis. A *Salmonella typhimurium* strain with the *asd* gene deleted must be supplied with diaminopimelate, without which the cells lyse (Galan et al. 1990). Thus, *HOM2* has been used as a selection marker in vaccine strains of *S. typhimurium* to ensure that plasmids, used to express desired antigens, are stably maintained (Galan et al. 1990; Kang et al. 2002). Loss of virulence was also found in *Legionella pneumophila* and *Staphylococcus aureus* when the gene coding for ASD was disrupted (Harb and Abu Kwaik 1998; Christian et al. 2002). The aspartate pathway in *Mycobacterium smegmatis* is essential, likely due to the need for biosynthesis of diaminopimelate, without which the cells lyse (Pavelka and Jacobs 1996).

Due to its importance to prokaryotes and lower eukaryotes, ASD has been the target of several inhibition studies. A series of aspartyl phosphonates and phosphoramidates were evaluated as inhibitors of the reverse reaction catalyzed by ASD, namely phosphorylation of aspartate-4-semialdehyde (Cox et al. 2002). Of these, a compound which mimics the substrate 4-phosphoaspartate by replacing the bridging oxygen to phosphate with a difluoromethylene was found to be the best inhibitor of ASD_{Ec} in the direction opposite to the metabolic direction. ASD_{Ec} has been shown to use arsenate and vanadate in place of phosphate in the reverse reaction but other oxyanions were inhibitory with periodate showing the greatest inhibition (Kish and Viola 1999). It should be noted that efforts have been directed at developing inhibitors to the enzymes of the lysine branch of the aspartate pathway in bacteria. In general, these efforts have not been particularly rewarding as compounds which inhibit the enzymes did not show activity against bacteria (Girodeau et al. 1986; Cox et al. 2000). However, one study, which used short peptides to ensure transport of the inhibitor into the cell, identified compounds with antibacterial activity which inhibited an enzyme in the bacterial lysine biosynthesis branch of the aspartate pathway (Berges et al. 1986).

1.6.3. Homoserine Dehydrogenases

Homoserine dehydrogenase (HSD) catalyzes the reduction of aspartate-4-semialdehyde to homoserine using either NADPH or NADH, Scheme 1.1. HSD is the target of the natural product HON, discussed above. The kinetic mechanism of *E. coli*

HSD in the bifunctional aspartate kinase I – homoserine dehydrogenase I (AKI-$HSDI_{Ec}$) was shown to be ordered bi bi, with NADPH binding followed by aspartate-4-semialdehyde and homoserine being released prior to the release of $NADP^+$ (Angeles and Viola 1990). HSD in AKI-$HSDI_{Ec}$ was found to use a preferred-order random kinetic mechanism, where the preference is for NADPH binding prior to aspartate-4-semialdehyde and homoserine releasing prior to $NADP^+$ (Wedler et al. 1992).

S. cerevisiae HSD (HSD_{Sc}) purified to homogeneity was found to be inhibited by threonine in a competitive manner and by methionine in a noncompetitive manner with K_i values of 117 and 139 mM, respectively (Yumoto et al. 1991). HSD_{Sc} was cloned and sequenced and found to code for a 359 amino acid protein with a predicted molecular weight of 38.5 kD (Thomas et al. 1993). The kinetic mechanism of HSD_{Sc} was determined to be an ordered bi bi mechanism where NAD(P)H binds, followed by aspartate-4-semialdehyde and homoserine is the first product released followed by $NAD(P)^+$ (Jacques et al. 2001a). The stereochemistry of the reaction was investigated and the pro-S hydride of NAD(P)H is transferred to aspartate-4-semialdehyde (Jacques et al. 2001a).

HSD_{Sc} has been crystallized in the apo-enzyme, with NAD^+ and with L-homoserine and 3-aminopyridine adenine dinucleotide, an inert NAD^+ analog (DeLaBarre et al. 1998). The crystal structures of HSD_{Sc} were determined from these three crystal forms, revealing that HSD_{Sc} uses a novel mechanism of hydride transfer

(DeLaBarre et al. 2000). Chemicals that modify histidines were found to inactivate HSD_{Sc}, with substrates providing protection. Of the two conserved histidines that were replaced with alanine, only one was shown to affect activity and the effect was due, not to removal of a binding or catalytically important residue, but to gross changes in the oligomeric state of the enzyme (Jacques et al. 2001b).

1.6.4. Homoserine *O*-Acetyl Transferase

Homoserine *O*-acetyl transferase (HSAT) is the first step in the aspartate pathway branch leading to the biosynthesis of methionine in fungi and some bacteria, but not plants. HSAT catalyzes the acetylation of homoserine using acetyl coenzyme A to make *O*-acetyl homoserine, Scheme 1.1. However, several bacteria, including *E. coli*, use an alternate mechanism to activate the C4 carbon of homoserine for nucleophilic attack in the subsequent step in the pathway, whereby succinyl CoA is used to acylate homoserine yielding *O*-succinyl homoserine catalyzed by homoserine transsuccinylase (HTS) (Born and Blanchard 1999). To date, no organism has been found to possess both acylation capacities (Born et al. 2000). In plants, the first intermediate in the biosynthesis of methionine is cystathionine, as opposed to *O*-acetyl homoserine in bacteria and fungi (Azevedo et al. 1997). In plants, cystathionine is made from *O*-phosphohomoserine, the product of homoserine kinase, using cystathionine-γ-synthase (Azevedo et al. 1997).

HTS from *E. coli* (HTS_{Ec}) was shown to use a ping pong kinetic mechanism where an enzyme nucleophile, likely Cys142, attacks succinyl CoA to form a succinyl-enzyme intermediate. The intermediate is attacked by the hydroxyl group of homoserine, which is likely deprotonated by an active site base, to give *O*-succinyl homoserine (Born and Blanchard 1999). Evaluation of the equilibrium constant showed that it favours the metabolic direction of acylating homoserine (Born and Blanchard 1999).

HSAT from *Haemophilus influenzae* ($HSAT_{Hi}$) was found to use a ping pong kinetic mechanism where acetyl CoA is used to first acetylate a nucleophile in the enzyme active site and then transfer this acetyl group to the hydroxyl group of homoserine (Born et al. 2000). Proposals were made regarding amino acids which could serve as an active site nucleophile, general base and a possible but not necessary general acid, based on conserved residues in $HSAT_{Hi}$ and precedents in other enzymes (Born et al. 2000). Similar to HTS_{Ec}, the equilibrium constant of $HSAT_{Hi}$ was found to favour the metabolic direction of acylating homoserine (Born et al. 2000). The *MET2* gene from *S. cerevisiae* was cloned by two independent groups, however, only one provided sequence data, which contained a sequencing error near the end of the gene causing them to misidentify the stop codon (Baroni et al. 1986; Langin et al. 1986).

1.6.5. Homoserine Kinase

Homoserine Kinase (HSK) catalyzes the first step in the aspartate pathway branch leading to biosynthesis of threonine and isoleucine, Scheme 1.1. HSK transfers the gamma phosphate of ATP to homoserine yielding ADP and *O*-phosphohomoserine.

E. coli HSK (HSK_{Ec}) in crude extracts was shown to be inhibited by L-Thr, with 2 mM reducing activity by 50%, and a K_i of approximately 1 mM with L-Thr acting as a competitive inhibitor versus homoserine (Theze et al. 1974). Highly purified HSK_{Ec} was found to be a dimer with monomeric molecular weights of 29 kD. K_m values for ATP and homoserine were found to be 550 μM and 240 μM, respectively, with L-Thr giving a K_i of 0.6 mM which was competitive versus homoserine (Burr et al. 1976). The order of substrate binding to HSK_{Ec} was found to be preferred order random, with ATP preferentially binding before homoserine (Shames and Wedler 1984). Chemical modification and pH studies have shown HSK_{Ec} to have a histidine and lysine that are important for catalytic activity (Huo and Viola 1996a). Site directed mutagenesis later revealed that Arg234 was important for binding homoserine, and His202 was involved in binding homoserine for substrate inhibition (Huo and Viola 1996b).

The gene for HSK from *S. cerevisiae* (HSK_{Sc}) was cloned and found to code for 356 amino acids giving a protein with a molecular weight of 40 kD, and the purified

enzyme was a homodimer (Mannhaupt et al. 1990). Approximately 10 mM L-Thr gives 50% inhibition of HSK_{Sc} (Ramos et al. 1991).

The crystal structure of HSK from *Methanococcus jannaschii* (HSK_{Mj}), which was determined in the absence of substrates or inhibitors, showed that HSK_{Mj} uses an atypical ATP binding motif (Zhou et al. 2000). Further crystal structures of HSK_{Mj} with various substrates, products, and inhibitors identified amino acids in the active site and revealed the absence of an active site base near the hydroxyl group of homoserine (Krishna et al. 2001).

1.6.6. Threonine Synthase

Threonine synthase (TS) is a pyridoxal phosphate dependent enzyme that catalyzes a β,γ-replacement reaction allowing *O*-phosphohomoserine to be converted to L-threonine, Scheme 1.1. Inhibition of *E. coli* TS (TS_{Ec}) by L-APPA which is found in the plumbemycins and rhizocticin natural products, has been examined (Laber et al. 1994). In addition, a slow, tight binding inhibitor of TS_{Ec} has been studied (Farrington et al. 1993).

TS from *S. cerevisiae* (TS_{Sc}) is encoded on the *THR*4 gene (Aas and Rognes 1990; Ramos and Calderon 1994). The crystal structure of the monomeric TS_{Sc} with

pyridoxal phosphate has been determined, which has permitted the modeling of *O*-phosphohomoserine into the active site (Garrido-Franco et al. 2002).

TS from *Arabidopsis thaliana* (TS_{At}) is a dimer and is regulated by AMP inhibition and SAM activation, a product of the methionine pathway which competes with TS for O-phosphohomoserine (Laber et al. 1999). The crystal structure of TS_{At} has been determined in the absence of substrates and allosteric effectors (Thomazeau et al. 2001).

1.7. General Mechanisms of Kinases

Kinases catalyze the transfer of the γ-phosphate from ATP to a substrate. This transfer can go by one of two common paths, Figure 1.3. One path is a direct transfer of the γ-phosphate from ATP to the substrate. The second is an indirect path where the γ-phosphate is transferred to the enzyme, forming a phosphoenzyme intermediate, the phosphate is then transfered to the substrate. The stability of phosphoenzyme intermediates can vary greatly (Thompson and Cole 2001).

These phosphoryl transfer paths can go through a range of mechanisms between two extremes, Figure 1.3. One extreme, is a fully dissociative mechanism that goes through a metaphosphate intermediate and resembles a unimolecular nucleophilic

substitution (S_N1) reaction. In such a mechanism there is contact, but no bonding, between the electron orbitals of the oxygen and phosphorus on both the leaving and entering sides of the metaphosphate (Mildvan 1997). The second extreme is a mechanism where there is bonding between the oxygen and phosphorus on both the leaving and entering sides, generating a pentavalent phosphorane intermediate. This extreme is called a fully associative mechanism and resembles a bimolecular nucleophilic substitution (S_N2) reaction (Mildvan 1997).

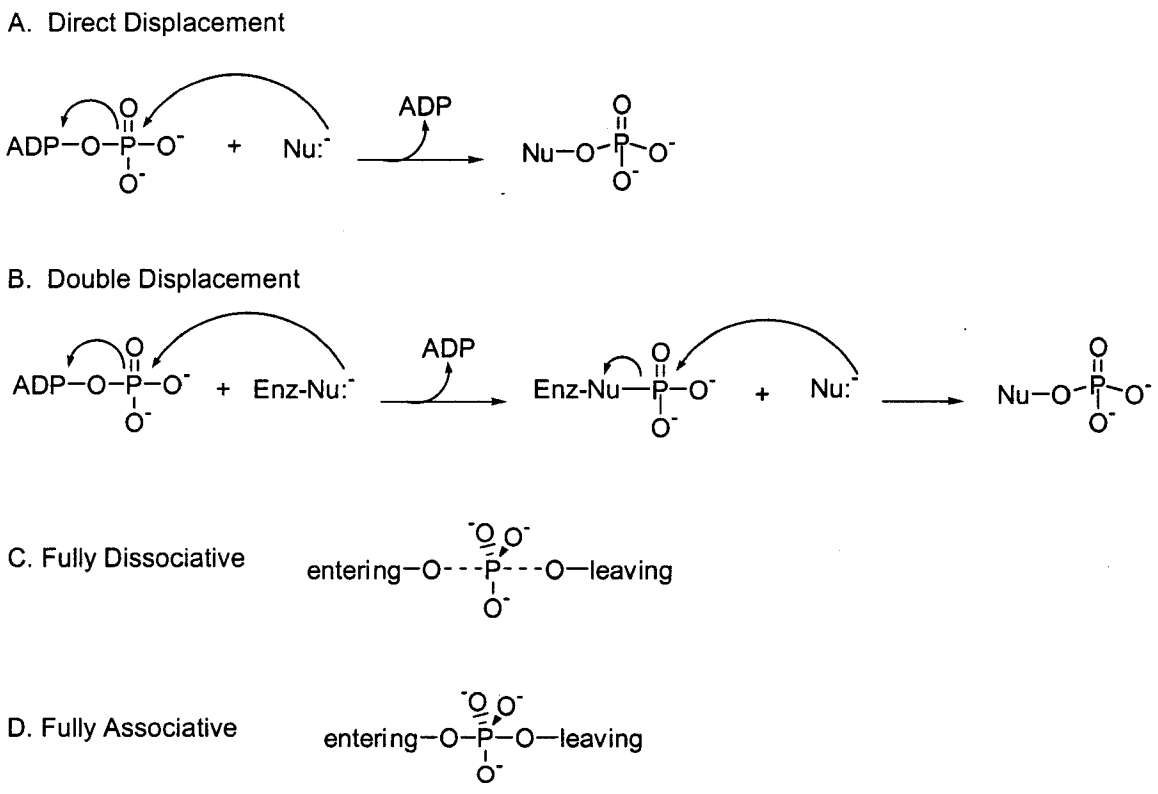


Figure 1.3. Phosphoryl transfers can go by two paths (A and B) using a mechanism falling at or between two extremes (C and D).

The two phosphoryl transfer paths can be differentiated in a number of ways. The initial velocity of a two substrate enzyme at various concentrations of the fixed substrate will show either parallel or intersecting lines in a double reciprocal plot. Parallel lines are indicative of a covalent intermediate, because such a pattern is diagnostic of a ping pong mechanism which goes through a covalent intermediate. In ping pong mechanisms, an enzyme binds a substrate, transfers a group from the substrate to the enzyme, and releases the first product to generate a stable enzyme form to complete the first half of the reaction. The second half involves binding a second substrate, transferring a group from the enzyme to the second substrate producing a product which is released. An intersecting pattern in an initial velocity experiment indicates a ternary complex is formed and does not rule out the possibility of a covalent intermediate. For example, in a ternary complex mechanism the two substrates could be bound to the enzyme and the phosphoryl group could be passed first to the enzyme and then to the second substrate, prior to release of the first product.

Another way of discerning whether a group transfer path goes through a covalent intermediate is to use a substrate where the group to be transferred is radiolabelled. In the case of phosphotransfer, if the phosphoenzyme intermediate is stable, one could use radioactively labelled ATP, where ^{32}P is in the γ-phosphate, to determine if the enzyme can be labelled. If the enzyme is labelled, it indicates either that the γ-phosphate has been transferred to the enzyme or that there was no transfer and the radiolabelled ATP is

simply tightly bound. These two possibilities could be distinguished by determining if ATP with ^{32}P in the non-transferred α-phosphate, also labels the enzyme, indicating that tight binding of ATP responsible. Further proof of a phosphoenzyme intermediate would come from purifying the stable phosphorylated enzyme and providing the second substrate to determine if the radioactive phosphate is transferred to the second substrate in a kinetically competent manner.

In the situation where the phosphoenzyme intermediate is unstable, one could look for hydrolysis of ATP to ADP in the absence of a second substrate. A lack of ATPase activity would either indicate that there is no hydrolysis of ATP or that hydrolysis occurs but the γ-phosphate reacts with the generated ADP to form ATP before ADP can leave the active site. If ATP is being regenerated, it would have to be regenerated 100% of the time, otherwise ATPase activity would be observed.

Another way to prove the presence of a covalent intermediate that garners additional information is to directly visualize it in crystal structures. As discussed above, one of the difficulties in determining the path of phosphoryl transfer is due to the instability of phosphoenzyme intermediates. This difficulty has been overcome in NMR solution structure (Cho et al. 2000) and crystal structure determination (Cho et al. 2001) of phosphoenzyme intermediates by the use of a phosphate mimic that can form similar bonds but has greater stability.

Determining what mechanism is used to transfer the phosphate between substrates requires the use of a positional isotope exchange (PIX) experiment. PIX experiments of kinases use a chemically synthesized ATP where the bridging oxygen between the β and γ-phosphates is enriched in ^{18}O (Midelfort and Rose 1976). If a metaphosphate is formed as a stable intermediate and the β-phosphate of ADP is free to rotate, the enzyme will scramble the labelled oxygen between the three possible positions in the β-phosphate when a second substrate is absent. This occurs because the enzyme is able to hydrolyze ATP forming ADP and the metaphosphate intermediate, in the absence of the second substrate the reaction can reverse direction using one of the oxygens of the β-phosphate of ADP as a nucleophile to attack the metaphosphate reforming ATP. If the β-phosphate of ADP is free to rotate, a new oxygen can form the bridge between the β and γ-phosphates in ATP two thirds of the time. The absence of scrambling of the ^{18}O label indicates either that a metaphosphate is not formed or that the β-phosphate of ADP is torsionally confined. The location of ^{18}O is determined using ^{31}P NMR since ^{18}O produces a 0.02 ppm upfield chemical shift per ^{18}O (Raushel and Villafranca 1988).

A new way to determine the mechanism of phosphoryl transfer involves direct visualization of the intermediate in crystal structures. Evidence for an associative transfer mechanism was found in the crystal structure of phosphorylated β-phosphoglucomutase - Mg^{2+} - glucose-1,6-(bis)phosphate complex which clearly showed the presence of a trigonal bipyramidal phosphorane intermediate (Lahiri et al. 2003).

Evidence for a dissociative transfer mechanism was found when a metaphosphate was observed in the crystal structure of fructose-1,6-bisphosphatase grown in the presence of an equilibrium mixture of substrates and products (Choe et al. 2003).

1.8. Exploring the Aspartate Pathway: Building on What is Known

The enzymes of the aspartate pathway have been studied for half a century. Much has been learned, but much remains to be discovered. For example, the kinases of the aspartate pathway, AK and HSK, are important control points of the pathway, yet the mechanism of how these enzymes work is not completely understood. This is particularly true for AK, for which a representative crystal structure has yet to be determined.

As discussed in detail above, the aspartate pathway should make an excellent target for agents directed against plants, bacteria, and fungi. Furthermore, the already great need for the development of new antifungal agent/target pairs is only intensifying, as the number of people with fungal infections increases and fungi develop mechanisms to resist the palette of antifungals currently used.

It was from this background that the following goals were developed. 1. Investigate the mechanism of phosphoryl transfer in AK. 2. Determine if inhibitors to

AK and HSK could be rationally designed. 3. Develop a method to screen enzymes in the aspartate pathway in the metabolic direction. 4. Determine if inhibitors to the enzymes in the aspartate pathway could be found in compound libraries using a high throughput screening format.

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Chapter 2

Identification of Amino Acids Important to Catalysis in *Saccharomyces cerevisiae* Aspartate Kinase

David C. Bareich, Gerard D. Wright

2.1. Preface

All experimental work reported in this paper was performed by David Bareich during his Ph.D. studies in the laboratory of Dr. G. Wright. The paper has been submitted to Protein Science, but, at the time of this writing, has not been accepted.

2.2. Abstract

The *HOM3* gene of *Saccharomyces cerevisiae* encodes aspartate kinase (AK_{Sc}), which phosphorylates L-Asp as the first step in the pathway for the biosynthesis of L-Thr, L-Met and L-Ile. Using site directed mutagenesis, we have evaluated the importance of residues in AK_{Sc} that are strongly conserved among aspartate kinases or in other small molecule kinases. Steady state kinetic analysis of the purified AK_{Sc} mutants reveals that many of the targeted amino acids have important roles in the enzymatic reaction, particularly K18 and H292. These conclusions were supported by studying the effect of pH on the steady state kinetic parameters of AK_{Sc} and the K18A, H292A, and H497A variants. These results provide the first identification of amino acid residues crucial to the action of this important metabolic enzyme.

Keywords: *Saccharomyces cerevisiae*, aspartate kinase, mutagenesis, steady state kinetics, pH effects

2.3. Introduction

Aspartate kinase, (AK), is the first enzyme in the aspartate pathway, which is responsible for the biosynthesis of L-Thr, L-Met, and L-Ile in fungi, and L-Thr, L-Met, and L-Lys in bacteria and plants. The pathway is not found in mammals, making it of interest as a potential target for new antibiotics. In both bacteria and fungi, the pathway is regulated by feedback inhibition of AK. *Saccharomyces cerevisiae* AK (AK_{Sc}) is regulated by L-Thr (Stadtman et al. 1961), however the situation is more complex in the bacterium *Escherichia coli* where there are three AKs that are regulated by feedback inhibition by three different pathway products. The first two AKs in *E. coli* are bifunctional enzymes with homoserine dehydrogenase, the first is regulated by L-Thr and the second by L-Met (Viola 2001). *E. coli* aspartate kinase III ($AKIII_{Ec}$) is monofunctional and is regulated by L-Lys (Funkhouser et al. 1974). A molecular understanding of how AK catalyzes the phosphorylation of L-Asp with the γ-phosphate of adenosine triphosphate (ATP) will aid in the design of new inhibitors to the enzyme, which could serve as leads for the generation of new antibiotics. There is currently very little mechanistic and structural information about AK to guide such efforts.

Primary sequence analysis of AK's reveals a conserved Amino Acid Kinase (AAK) domain and two ACT domains, ACTI and ACTII, Figure 2.1. The AAK domain in AK_{Sc}, is conserved among many kinases including acetylglutamate kinase, carbamate kinase, and uridylate kinase (Bateman et al. 2002). The two ACT domains are named for

aspartate kinase, chorismate mutase, and TyrA, the regulator for the L-Tyr and phenol metabolism operons (Aravind and Koonin 1999). ACT domains are proposed to be a conserved regulatory binding fold, with pairs of ACT domains specifically binding an amino acid leading to regulation of the enzyme (Chipman and Shaanan 2001). To date efforts to experimentally distinguish important amino acids in AK have only identified those that reduce or eliminate allosteric regulation by the products of the aspartate pathway, for example the intracellular concentration of L-Thr increases 8 times for AK_{Sc}-G452D (Martin-Rendon et al. 1993).

With this in mind, we used two strategies to formulate hypotheses about amino acids in AK_{Sc} that might be important for substrate binding and catalysis. Since there are, as yet, no AK crystal structures, identification of amino acids important for substrate binding, catalysis, and allosteric regulation can only be guided by primary sequence alignments and fundamental biochemical studies. We used primary sequence alignments to identify strongly conserved amino acids in known AKs and identified a motif common among AKs. In addition, we compared the AK primary sequences with those of other small molecule kinases that have been characterized to a greater extent or for which crystal structures complexed with substrates are available. Using a site directed mutagenesis approach, we have identified K18 and H292 in the AAK domain as important residues in catalysis and E279 as important in L-Thr inhibition.

2.4. Results and Discussion

2.4.1. Expression and Purification of AK_{Sc}

The yield of recombinant AK_{Sc} expressed heterologously in *E. coli* BL21(DE3)/ pET28+AK_{Sc} was found to be greatest when the induction temperature was reduced to 16°C. Purification by way of a combined Ni affinity and Q Sepharose anion exchange step followed by a Superdex S75 gel filtration step was found to yield 5.8 mg of protein with an n fold purification of 230 times, Table 2.1.

2.4.2. Analysis of AK_{Sc} Engineered Variants

2.4.2.1. Signature Motif for AKs

An amino acid sequence alignment created with Clustal W (Thompson et al. 1994) of AKs shows a unique motif, VxKFGG(T/S)SV, proximal to the N terminus, Figures 2.1 and 2.2, which we call the AK Signature Motif. We chose to investigate the effect of exchanging K18 (AK_{Sc} numbering) with Ala, Arg, and Gln; T22 with Ala; and S23 with Ala on the steady state kinetic parameters of AK_{Sc} to assess the importance of these strongly conserved residues.

The AK_{Sc} variants K18A, K18R, and K18Q all have greatly reduced k_{cat} values, in the range of 82 to 160 times decreased for either substrate, Table 2.2, suggesting that K18 is important for catalysis. K_m values for both substrates are essentially at wild type levels for K18A, K18Q, and K18R with the exception of K18R ATP K_m which is decreased 2.9 fold. There is surprisingly little difference in the conservative replacement of K18 with Arg when compared to the nonconservative replacement with Gln where charge is lost and Ala where charge and size are lost. These K18 substitutions gave the greatest k_{cat} decreases of all variants studied, further suggesting that K18 makes an important catalytic contribution. The k_{cat} differences cause the k_{cat}/K_m values to be reduced between 55 to 80 times for L-Asp and 55 to 110 times for ATP, suggesting that K18 may also have a role in substrate recognition. The L-Thr IC_{50} values are essentially the same as wild type for K18A, K18Q, and K18R.

All steady state kinetic parameters for T22A are similar to wild type with the exception of ATP K_m and k_{cat}/K_m values which increased 4.2 and decreased 3.6 times, respectively. S23A differs significantly only in k_{cat}/K_m which is decreased 4.0 and 3.7 times for L-Asp and ATP, respectively. The simplest interpretation of these data is that T22 and S23 do not have important functions in AK_{Sc}. The L-Thr IC_{50}'s for T22A and S23A are at the wild type level.

2.4.2.2. AK_{Sc}-Histidine 497

AK_{Sc}-H497 was found to be highly conserved among AKs (20 of 28 sequences), Figure 2.3. Previous diethyl pyrocarbonate inactivation studies and pH profiles of the *E. coli* AK functionality in the bifunctional aspartate kinase I – homoserine dehydrogenase I ($AKI\text{-}HSDI_{Ec}$) enzyme and $AKIII_{Ec}$, suggested a His residue is important for activity (Angeles et al. 1989; Keng and Viola 1996). Similar studies in our own lab suggest the same is true for AK_{Sc} (data not shown). To investigate the importance of this residue, a variant was made where H497 was replaced with Ala.

H497A has little effect on K_m for either substrate, but k_{cat} is decreased 6.7 fold for both substrates, while k_{cat}/K_m decreased 13 and 11 times for L-Asp and ATP, respectively. The L-Thr IC_{50} is unchanged compared to wild-type. Since the steady state kinetic parameters for both substrates are effected similarly, it is difficult to suggest a function for H497 beyond a general involvement in substrate capture and catalysis.

2.4.2.3. Amino Acid Similarity Between AKs and Homoserine Kinases

AK and homoserine kinase (HSK) catalyze similar phosphotransfer reactions with substrates that differ only in the oxidation state of the phosphate-accepting carbon. Crystal structures of *Methanococcus jannaschii* HSK (HSK_{Mj}) have been solved with ADP and also with L-homoserine plus the ATP analog 5'adenylylimidotriphosphate

(Zhou et al. 2000; Krishna et al. 2001). These structures identify amino acids in the active site of HSK_{Mj} that are important for substrate binding. Global primary sequence alignments of HSKs and AKs do not show large regions of similarity. However, an alignment of several AKs and HSKs, Figure 2.4, shows that amino acids found in the active site of HSK_{Mj} align with similar or identical amino acids in AK_{Sc}.

Specifically, HSK_{Mj}-E130 interacts with the Mg^{2+} bound between the β and γ-phosphates of ATP and is suggested to activate the γ-phosphate of ATP (Krishna et al. 2001). This residue is conserved among several HSKs as well as members of the galactokinase, homoserine kinase, mevalonate kinase, and phosphomevalonate kinase (GHMP) superfamily (Bork et al. 1993). In Figure 2.4, AK_{Sc}-E279 aligns with HSK_{Mj}-E130, which leads to the hypothesis that AK_{Sc}-E279 may be involved in ATP binding and or activation of the γ-phosphate of ATP. The importance of AK_{Sc}-E279 is further underscored by its strong conservation among AKs.

AK_{Sc}-E279A shows wild type K_m values and is 47 and 44 times decreased in k_{cat} for L-Asp and ATP, respectively. The k_{cat}/K_m values decrease by 24 and 68 times for L-Asp and ATP, respectively. These results support the hypothesis that E279 may play an important role in substrate capture, particularly for ATP, as well as suggesting a role in catalysis. The results also suggest that E279 likely has a role in allosteric inhibition as the L-Thr IC_{50} increased 14 times, Table 2.2, and the L-Thr K_i values increase 7.4 and 8.2

times with respect to L-Asp and ATP in E279A compared to wild type, Table 2.3. The mechanism of L-Thr inhibition with respect to ATP changes from mixed inhibition in AK_{Sc} to uncompetitive inhibition in E279A. The observed allosteric inhibition changes were surprising because E279 is not located near either of the two ACT domains in the primary sequence, Figure 2.1.

The side chain oxygen of HSK_{Mj}-N141 forms a hydrogen bond with the δ-OH of L-homoserine and is strongly conserved among HSKs (Krishna et al. 2001). Figure 2.4 shows HSK_{Mj}-N141 aligning with AK_{Sc}-T295. In general, AK_{Sc}-T295 is poorly conserved among AKs with several sequences having Ala or Met in this position, however, Thr is found in this position in all three *E. coli* AKs as well as in *Schizosaccharomyces pombe* AK. This position is poorly conserved across the GHMP superfamily with the exception of an invariant Asp found in mevalonate kinases. In human mevalonate kinase the Asp at this position is crucial for activity, and has been suggested to be a catalytic base (Potter and Miziorko 1997). Crystal structures of mevalonate kinase from *M. jannaschii* (Yang et al. 2002) and rat (Fu et al. 2002) have confirmed appropriate positioning of this residue for such a function. The differences in amino acid conservation of this position may be attributable to the differences in substrates and/or catalytic mechanism. Substrates that are phosphorylated on carboxylic acids (pKa<5), such as Asp, are largely deprotonated at physiological pH, whereas alcohols (pKa≈15) are not. Thus, alcohols generally will need a catalytic base to deprotonate them for the nucleophilic attack of the γ-phosphate of ATP, while carboxylic

acids will not. This could explain the poor conservation at this position in AKs, however, AK_{Sc}-T295 could have a role in positioning L-Asp. To test the importance of AK_{Sc}-T295 the appropriate mutation was made to exchange isosteric Val for T295.

AK_{Sc}-T295V steady state parameters are similar to wild type, with the exception of a 6.7 times decrease in k_{cat}/K_m for ATP. These results do not support a role in L-Asp recognition, but may suggest a role in ATP capture.

HSK_{Mj}-H138 adopts multiple conformations in the various crystal structures with one conformation, "close-in", allowing the side chain to interact with the phosphates of ATP through a water molecule (Krishna et al. 2001). HSK_{Mj}-H138 is conserved among homoserine kinases but not among other members of GHMP superfamily. Chemical modification studies and pH profiles have shown that *E. coli* HSK (HSK_{Ec}) has an important His residue (Huo and Viola 1996a). HSK_{Ec}-H139 which aligns with HSK_{Mj}-H138 was changed to Leu and found to alter the active site allowing entry of water which strongly competes with L-homoserine to be the phosphoryl acceptor as well as decreasing k_{cat} by 35 times compared to wild type (Huo and Viola 1996b). Figure 2.4 shows HSK_{Mj}-H138 aligning with AK_{Sc}-H292 a residue that is only partially conserved among AKs. We changed H292 to Ala and Gln to test the hypothesis that AK_{Sc}-H292 is involved in catalysis.

Neither AK_{Sc}-H292A nor H292Q show significant K_m changes, with the exception of a 4.5 times increase for ATP in H292A. The observed changes in k_{cat} are much more significant, particularly in H292A for ATP which showed a decrease of 120 times, resulting in a 550 times decrease in k_{cat}/K_m compared to wild type. The changes observed for L-Asp steady state kinetic parameters were significant, but far less substantial compared to the ATP effects. Although the observed effects are similar for the two variants, in all cases the Ala variant showed larger effects, indicating that Gln can replace some of the function of His, and that the remaining function may be attributable to the ionization of His. These variants show effects that are similar in magnitude to those observed for the HSK_{Ec}-H139L change (Huo and Viola 1996b). These results support the hypothesis that H292 is involved in catalysis, as well as suggesting a role in ATP binding. The L-Thr IC_{50} values were similar to wild type for both variants.

HSK_{Mj}-R235 forms a salt bridge with the carboxylate of L-homoserine and is strongly conserved among HSKs. Figure 2.4, shows HSK_{Mj}-R235 aligning with AK_{Sc}-R419, an amino acid that is only partially conserved among AKs. When the similar position in the *E. coli* enzyme, HSK_{Ec}-R234, was exchanged with Leu, the L-homoserine K_m increased 285 times and k_{cat} decreased 92 times compared to wild type (Huo and Viola 1996b). From this, we hypothesized that AK_{Sc}-R419 may be involved in L-Asp binding. To test this, we assessed the effect of exchanging Ala for AK_{Sc}-R419.

AK-R419A showed a 10 and 8.9 times decrease in k_{cat}, and 7.1 and 29 times decrease in k_{cat}/K_m for L-Asp and ATP, respectively. These results suggest R419 is important to AK_{Sc}, particularly for ATP capture, but do not support the hypothesis that R419 is involved in L-Asp binding. The L-Thr IC_{50} value was similar to wild type.

2.4.2.4. Amino Acid Similarity Between AK_{Sc} and *N*-Acetylglutamate Kinase

In the recent crystal structure of the AAK domain protein, *E. coli N*-acetylglutamate kinase ($NAGK_{Ec}$), D181 is shown to form a hydrogen bond to the 3'-OH of ATP, this residue is well conserved in other kinases of the AAK family (Ramon-Maiques et al. 2002). Alignment of $NAGK_{Ec}$, AK_{Sc}, AKI-$HSDI_{Ec}$, and $AKIII_{Ec}$, aligns $NAGK_{Ec}$-D181 with AK_{Sc}-E254, Figure 2.5. From this we hypothesize that AK_{Sc}-E254 is equivalent to $NAGK_{Ec}$-D181 and is involved in binding ATP. To test this hypothesis the appropriate mutation was made to replace E254 with Ala.

E254A showed a decrease in k_{cat} of 9.1 and 11 times and k_{cat}/K_m of 19 and 11 times for L-Asp and ATP, respectively. Similar to R419A, these data suggest E254 is important to AK_{Sc} but do not readily support our initial hypothesis that E254 is involved in ATP binding, and point to diversity in amino acid roles in the AAK domain of AKs.

2.4.3. Effect of pH on the Steady State Kinetic Parameters of AK_{Sc} and K18A, H292A, and H497A Variants

The effect of pH on the steady state kinetic parameters of AK_{Sc} and select engineered variants was investigated to gain a better understanding of ionizations that are important for substrate capture and catalysis with respect to both substrates. Figure 2.6 shows plots of Log k_{cat} and Log k_{cat}/K_m versus pH with respect to both substrates. The AK_{Sc} Log k_{cat} and Log k_{cat}/K_m profiles for both substrates are similar, showing two ionizations are important for substrate capture and catalysis with the acidic ionization falling in the range of 5.1 to 6.2 and the basic between 9.1 and 9.8, Figure 2.6 and Table 2.4. The acidic ionization could reflect a His, while the basic ionization could be due to Tyr or Lys. The fact that the Log k_{cat} and Log k_{cat}/K_m profiles are similar indicates that substrate capture is not pH dependent and the reaction requires the two observed ionizable groups to be in particular protonation states (Cleland 1982). These results and conclusions are in reasonable agreement with those of the AK portion of AKI-$HSDI_{Ec}$ with respect to L-Asp Log V/K which showed ionizations at 6.9 and 9.8 that were attributed to His and Cys or Tyr, respectively (Angeles et al. 1989). They are also similar to those of $AKIII_{Ec}$ which showed ionizations of 6.3 and 9.5 for Log V/K for L-Asp and 5.8 and 9.8 for Log V which were attributed to His and Cys or Tyr, respectively (Keng and Viola 1996).

Analysis of pH titrations of variants entails comparison to titrations of the wild type enzyme. One hopes for a simple case where, for example, an acidic amino acid in the wild type is exchanged for a non-ionizable amino acid in the variant and the variant titration shows a loss of a kinetic decrease in the acidic region that is present in the wild type titration. When the simple case is not observed it can be very difficult to explain the data. The situation could be very complex and involve indirect interactions between the amino acid in question and one which is involved in the kinetic parameter being titrated. Without further information, such as crystal structures, such complex indirect interactions cannot be defined.

Compared to pH titrations on the wild type enzyme, titrations on the K18A, H292A, and H497A variants showed that two ionizations are found in all Log k_{cat} plots similar to the wild type enzyme, whereas only a single ionization could be found in several of the Log k_{cat}/K_m plots where two ionizations were found in the wild type. Specifically the basic ionization is lost in the K18A and H497A plots versus L-Asp, while the acidic ionization is lost in H292A plot versus L-Asp and in K18A versus ATP, Figure 2.6. While the loss of the acidic ionization in the Log k_{cat}/K_m plot for K18A versus ATP is not yet deciphered, the fact that the acidic ionization is lost for H292A versus L-Asp suggests that H292 needs to be deprotonated for AK_{Sc} L-Asp capture. Similarly for the basic ionization, its loss in the Log k_{cat}/K_m plot for H497A is noted, however the loss for K18A suggests that K18 needs to be protonated for AK_{Sc} L-Asp capture.

2.5. Materials and Methods

2.5.1. Cloning of AK_{Sc}

AK_{Sc} was PCR amplified from *S. cerevisiae* genomic DNA using the oligonucleotide primers 5'-CCCACGGCTAGCATGCCAATGGATTTCCAACC and 5'-CCGAATTCGGATCCTCATTAAATTCCAAGTCTTTTCAATTG. PCR reactions consisted of 50 pg genomic DNA, 10 pmol of each primer, Stratagene Thermopol buffer, 0.4 mM dNTP's, 100 mM $MgSO_4$, and 2 U Vent DNA polymerase. Reactions underwent 30 temperature cycles consisting of 94°C, 40°C, 72°C for 1, 2, and 1.3 min, respectively. The gene was cloned using standard techniques into the *Nhe* I and *Bam* HI restriction enzyme sites in the expression vector pET28 to create pET28+AK_{Sc} which was confirmed by DNA sequencing to be free of mutations.

2.5.2. Mutagenesis of pET28+AK_{Sc}

A Quikchange (Stratagene) PCR mutagenesis strategy was used to mutate codons in *HOM3* to create specific amino acid substitutions in AK_{Sc}. Amino acids to be targeted for substitution were identified from amino acid sequence alignments created with Clustal W (Thompson et al. 1994) of AK_{Sc} with other AKs and with other small molecule kinases. PCR reactions consisted of 50 ng of pET28+AK_{Sc}, 10 pmol of mutagenesis primers a and b for each mutation, Table 2.5, Stratagene native Pfu buffer, 250 μM

dNTP's, and 1 μL native Pfu DNA polymerase in a total volume of 25 μL. The reactions underwent 18 temperature cycles consisting of 95, 50, 68 °C for 1, 1, and 20 min, respectively. Following thermocycling, the entire reaction was digested with 20 U of *Dpn* I for 1 h at 37 °C. One sixth of this solution was used to transform *E. coli* BL21(DE3) cells by electroporation. Transformants were selected on 50 μg/mL kanamycin agar plates and the correct mutation was identified by DNA sequencing. The remainder of the *HOM3* gene sequence was confirmed to be correct in all cases.

2.5.3. Wild-type and Mutant AK_{Sc} Expression and Purification

AK_{Sc} with an N terminal hexa-His tag was expressed in 1 L LB cultures of *E. coli* BL21(DE3)/pET28+AK_{Sc}, which were grown from a 1% inoculum to an OD_{600} of 0.6 at 37°C. The cultures were cooled in an ice/water bath for 5 min, and induced with a final concentration of 1 mM isopropyl β-D-1-thiogalactopyranoside overnight in a 16 °C shaker. The cells from three to four 1 L cultures were combined and harvested by centrifugation at 13 000 x g for 10 min, and resuspended in ≈40-50 mL of 20 mM HEPES pH 8.0. Cells were disrupted with three passes through a French Pressure cell at 10 000 psi in the presence of 1 mM phenylmethylsulfonyl fluoride to inhibit serine proteases as well as 0.5 mg DNase and 0.5 mg RNase to reduce the viscosity caused by DNA and RNA. Cell debris was immediately pelleted by centrifugation at 43 000 x g for 10 min. The supernatant was passed over a 1-2 mL Ni-NTA column followed by 20 mM

HEPES pH 8.0 to wash the column until OD_{280} decreased to less than 100 mAU. The bound AK_{Sc} was eluted with 10 mL 250 mM imidazole + 20 mM HEPES pH 8.0 directly onto a 2 mL Q Sepharose column. The Q Sepharose column was washed with 20 mM HEPES pH 8.0 until the OD_{280} fell below 100 mAU and the bound AK_{Sc} was eluted using a linear gradient of 0 to 400 mM NaCl in the same buffer. Fractions containing AK_{Sc} were identified by sodium dodecyl sulfate – polyacrylamide gel electrophoresis (SDS-PAGE) and enzyme activity assays where possible. These were pooled, concentrated using a centrifugal concentrator and/or a speedvac without freezing the protein, and applied to a 60 cm Superdex S75 gel filtration column equilibrated and eluted with 200 mM NaCl + 20 mM HEPES pH 8.0. The initial fractions in the AK_{Sc} peak were found to be pure by inspection of Coomassie Blue stained SDS-polyacrylamide gels. All AK_{Sc} variants purified were obtained in similar yields and had similar chromatographic properties on the gel filtration column. Furthermore, AK_{Sc} and the variants gave similar fragments, as analyzed by SDS-PAGE, following subtilisin digestions, suggesting that the variants had the same global folding.

2.5.4. AK Assay and Determination of Steady State Kinetic Parameters

AK_{Sc} activity was determined by coupling production of adenosine dinucleotide (ADP) with the lactate dehydrogenase dependent oxidation of reduced nicotinamide adenine dinucleotide (NADH) in the presence of phosphoenol pyruvate and pyruvate kinase. Reaction mixtures contained approximately 0.2 μg AK_{Sc} (more for mutants with

reduced k_{cat} values), 7 mM ATP, 0.33 U pyruvate kinase, 0.66 U lactate dehydrogenase, 2.5 mM phosphoenol pyruvate, 1 mM NADH, 10 mM KCl, 40 mM $MgSO_4$, and 100 mM HEPES pH 7.5 in a final volume of 100 µL in a 96 well flat bottom microtitre plate. A 6 minute pre-incubation was used to ensure that any contaminating ADP was regenerated to ATP as well as allowing for temperature equilibration to 30 °C. The reactions were initiated with the addition of 30 mM L-Asp and monitored at 340 nm in a Spectramax microtitre platereader (Molecular Devices, Sunnyvale, CA, USA). For steady state kinetics, eight substrate concentrations in duplicate were used, ranging from 0.4 to 7 mM for ATP and 0.5 to 30 mM L-Asp. Data were fit to eq (1) for Michaelis-Menten kinetics using GraFit (Leatherbarrow 2001).

$$\upsilon = \frac{V_{\max} S}{(K_m + S)} \qquad (1)$$

2.5.5. L-Thr IC_{50} Determinations

Assays were similar to AK assays with the addition of L-Thr concentrations ranging from 0.05 to 150 mM, ATP and L-Asp were present at 5 and 12.5 mM, respectively. Data were fit to the four parameter eq (2), using GraFit (Leatherbarrow 2001) to solve for the IC_{50}, where A = minimum response plateau, D = maximum response plateau, I = concentration of inhibitor, and S = slope factor.

$$y = \frac{A - D}{1 + \left(\frac{I}{IC_{50}}\right)^{s}} + D \qquad (2)$$

2.5.6. pH Experiments

The effect of pH on the steady state kinetic parameters of AK_{Sc} and K18A, H292A, and H497A variants was determined using the AK assay. Steady state kinetic parameters were determined as previously stated with the exceptions that double the amount of pyruvate kinase – lactate dehydrogenase was used and the 100 mM HEPES pH 7.5 buffer was exchanged for a buffer mixture 100 mM in each of HEPES, morpholinoethanesulfonic acid, [(2-hydroxy-1,1-bis(hydroxymethyl)ethyl)amino]-1-propanesulfonic acid, and 2-(cyclohexylamino)ethanesulfonic acid at pH values ranging from 5.5 to 9.5 in 0.5 intervals. The buffer mixture was used to ensure that ionic strength was constant at all pH values. The pyruvate kinase – lactate dehydrogenase coupling enzymes were found to have little activity at pH values above 9.5. The log of k_{cat} or k_{cat}/K_m was plotted against pH and fit by nonlinear least fit squares regression using GraFit (Leatherbarrow 2001) to eq (3) for a single observed ionization where the y values vary from a limit at low pH to a second limit at high pH, eq (4) for a single observed ionization where the y values vary from a limit at low pH to zero at high pH, or eq (5) for two observed ionizations where the y values vary from a limit at low pH to a limit at

medium pH and a third limit at high pH. To allow such fitting, the units of k_{cat} were set to ms^{-1} in all instances to ensure the Log values were greater that 1.

$$y = \frac{Limit_{low} + Limit_{high} \times 10^{(pK_a - pH)}}{10^{(pH - pK_a)} + 1} \quad (3)$$

$$y = \frac{Limit \times 10^{(pK_a - pH)}}{10^{(pK_a - pH)} + 1} \quad (4)$$

$$y = \frac{(Limit_{low} + Limit_{medium}) \times 10^{(pH - pK_a 1)}}{10^{(pH - pK_a 1)} + 1} - \frac{(Limit_{medium} + Limit_{high}) \times 10^{(pH - pK_a 2)}}{10^{(pH - pK_a 2)} + 1} \quad (5)$$

2.6. Conclusion

From primary sequence alignments with AKs and other small molecule kinases, we have made hypotheses about what amino acids in AK_{Sc} are important for binding and catalysis. These hypotheses were tested by changing particular amino acids by way of site directed mutagenesis of *HOM3* and assessing the effect on the AK_{Sc} steady state kinetic parameters for both substrates as well as on the L-Thr IC_{50}. In general, the engineered variants were found to have altered steady state kinetic parameters when compared to the wild type enzyme. We have identified K18, which is located in the AK signature motif, as important for both substrate binding and catalysis, and H292, found in

the AAK domain, to play a role in ATP binding and catalysis. The results presented here give the first identification of amino acids important to AK_{Sc}, an enzyme which is vital for the biosynthesis of L-Thr, L-Met, and L-Ile.

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2.8. References

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Table 2.1. Purification of AK_{Sc} from 4 L of *E. coli* BL21(DE3)/ pET28+AK_{Sc}

Step	Total Protein (mg)	Activity (nmol/s)	Specific Activity (nmol/s*mg)	Purification (n fold)	Recovery (%)
Cell Extract	1400	0.18	.00013	1.0	100
Supernatant Combined Ni Affinity – Q Sepharose Anion Exchange	11	0.083	.0074	57	42
Superdex S75 Gel Filtration	5.8	0.17	.029	230	16

Table 2.2. Steady state kinetics for AK_{Sc} and engineered variants. Errors listed for K_m and k_{cat} are exclusively errors of data fitting.

Enzyme	Varied Substrate	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($M^{-1}s^{-1}$)	$\frac{k_{cat_{WT}}}{k_{cat_{mutant}}}$	$\frac{k_{cat}/K_{m_{WT}}}{k_{cat}/K_{m_{mutant}}}$	L-Thr IC_{50} (mM)
WT	L-Asp	4.59 ± .51	46.6 ± 1.54	10 200			2.7 ± .09
K18A	L-Asp	3.21 ± .39	0.414 ± 0.02	129	110	79	4.2 ± .73
K18R	L-Asp	1.63 ± .14	0.300 ± 0.01	184	160	55	6.8 ± .58
K18Q	L-Asp	4.25 ± .72	0.543 ± 0.03	127	86	80	4.5 ± .22
T22A	L-Asp	7.08 ± .78	45.0 ± 1.76	6 350	1.0	1.6	3.1 ± .24
S23A	L-Asp	6.99 ± .71	17.9 ± 0.70	2 560	2.6	4.0	3.8 ± .26
H497A	L-Asp	8.66 ± .50	6.92 ± 0.15	797	6.7	13	4.5 ± .28
E279A	L-Asp	2.33 ± .33	0.983 ± 0.03	421	47	24	38 ± 4.9
T295V	L-Asp	3.19 ± .40	18.0 ± 0.54	5 640	2.6	1.8	7.9 ± .67
H292A	L-Asp	6.81 ± .95	2.49 ± 0.13	366	19	28	5.2 ± .28
H292Q	L-Asp	11.9 ± .95	10.3 ± 0.35	864	4.5	12	5.3 ± .15
R419A	L-Asp	3.25 ± .39	4.65 ± 0.14	1 430	10.0	7.1	4.1 ± .09
E254A	L-Asp	9.77 ± 1.11	5.14 ± 0.23	526	9.1	19	3.9 ± .23
WT	ATP	0.740 ± .05	49.3 ± 1.39	66 600			
K18A	ATP	0.887 ± .12	0.534 ± 0.02	602	92	110	
K18R	ATP	0.255 ± .04	0.310 ± 0.01	1 220	160	55	
K18Q	ATP	0.614 ± .09	0.600 ± 0.02	977	82	68	
T22A	ATP	3.10 ± 0.38	56.9 ± 2.65	18 300	0.87	3.6	
S23A	ATP	1.05 ± .11	19.1 ± 0.56	18 200	2.6	3.7	
H497A	ATP	1.26 ± .11	7.33 ± 0.17	5 820	6.7	11	
E279A	ATP	1.15 ±.23	1.13 ± 0.08	983	44	68	
T295V	ATP	2.35 ± .68	23.3 ± 3.13	9 910	2.1	6.7	
H292A	ATP	3.36 ± .41	0.406 ± 0.20	121	120	550	
H292Q	ATP	1.45 ± .10	8.30 ± 0.22	572	5.9	120	
R419A	ATP	2.40 ± .52	5.55 ± 0.30	2 310	8.9	29	
E254A	ATP	0.700 ± .09	4.43 ± 0.14	6 330	11	11	

Table 2.3. L-Thr K_i values and mechanism of inhibition for AK_{Sc} and AK_{Sc}-E279A.

Enzyme	Varied Substrate	Mechanism of Inhibition	K_i (mM)	K_i' (mM)
AK_{Sc}	L-Asp	Mixed	1.9 ± 0.37	7.6 ± 2.0
AK_{Sc}-E279A	L-Asp	Mixed	14 ± 3.6	60 ± 10
AK_{Sc}	ATP	Mixed	3.4 ± 0.78	6.7 ± 1.4
AK_{Sc}-E279A	ATP	Uncompetitive	28 ± 1.4	

Table 2.4. Summary of pH effects on the steady state kinetic parameters of AK_{Sc} and select engineered variants as well as the equation used to fit the data shown in Figure 2.6.

		k_{cat}			k_{cat}/K_m		
Enzyme	Varied Substrate	eq	pK_1	pK_2	eq	pK_1	pK_2
WT	L-Asp	5	5.3 ± .54	9.3 ± .28	5	5.7 ± .22	9.5 ± .25
K18A	L-Asp	5	6.5 ± .36	7.7 ± .16	3	6.0 ± .080	
H292A	L-Asp	5	6.1 ± .74	10.3 ± .66	4		10.3 ± .080
H497A	L-Asp	5	5.7 ± .31	8.1 ± .17	3	6.9 ± .13	
WT	ATP	5	5.1 ± .51	9.8 ± .42	5	6.2 ± .017	9.1 ± .025
K18A	ATP	5	7.0 ± .35	8.6 ± .36	3		8.7 ± .33
H292A	ATP	5	6.2 ± .023	9.6 ± .026	5	6.2 ± .20	8.9 ± .23
H497A	ATP	5	6.0 ± .16	7.7 ± .076	5	6.0 ± .093	8.8 ± .18

Table 2.5. PCR primers used for site directed mutagenesis of AK_{Sc}.

AK-K18Aa	5'-CTGGGTCGTGCAAGCGTTCGGTGGTACATC
AK-K18Ab	5'-GATGTACCACCGAACGCTTGCACGACCCAG
AK-K18Ra	5'-GATGTACCACCGAACCTTTGCACGACCC
AK-K18Rb	5'-GGGTCGTGCAAAGGTTCGGTGGTACATC
AK-K18Qa	5'-GATGTACCACCGAACTGTTGCACGACCC
AK-K18Qb	5'-GGGTCGTGCAACAGTTCGGTGGTACATC
AK-T22Aa	5'-GAAATTTACCGACCGAAGCACCACCGAACTTTTG
AK-T22Ab	5'-CAAAAGTTCGGTGGTGCTTCGGTCGGTAAATTTC
AK-S23Aa	5'-GGAAATTTACCGACAGCTGTACCACCGAAC
AK-S23Ab	5'-GTTCGGTGGTACAGCTGTCGGTAAATTTCC
AK-E254Aa	5'-GTAAATATACCATCAACTGCCTTCCAAACTTGTAG
AK-E254Ab	5'-CTACAAGTTTGGAAGGCAGTTGATGGTATATTTAC
AK-E279Aa	5'-GTTAATTCAGAAGCTGCTTCTGGAGTAACAC
AK-E279Ab	5'-GTGTTACTCCAGAAGCAGCTTCTGAATTAAC
AK-H292Qa	5'-GGTTCCGAAGTTATACAACCTTTTACGATGGAAC
AK-H292Qb	5'-GTTCCATCGTAAAAGGTTGTATAACTTCGGAACC
AK-T295Va	5'-CTAATAACTTGTTCCATCACAAAAGGATGTATAAC
AK-T295Vb	5'-GTTATACATCCTTTTGTGATGGAACAAGTTATTAG
AK-R419Aa	5'-CAATTTTTCCTCAGCTTGTGCCAGAGATTTTAATG
AK-R419Ab	5'-CATTAAAATCTCTGGCACAAGCTGAGGAAAAATTG
AK-H497Aa	5'-CCATAAAAGCGCTACAATGTATTGCTGCCAAGTTACTAAGTGAG
AK-H497Ab	5'-CTCACTTAGTAACTTGGCAGCAATACATTGTAGCGCTTTTATGG

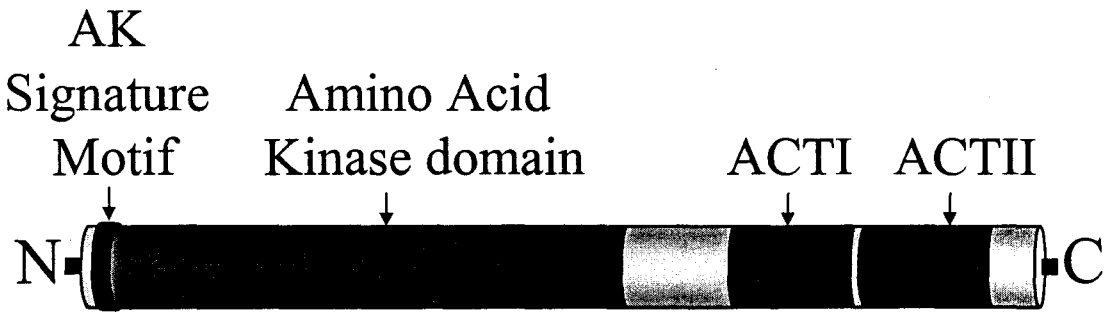

Figure 2.1. Schematic of the primary sequence of AK_{Sc} showing the AK Signature Motif, (amino acids 15-23) (see results and discussion), Amino Acid Kinase domain, (13-310), and two ACT domains, ACTI (363-433) and ACTII (438-506).

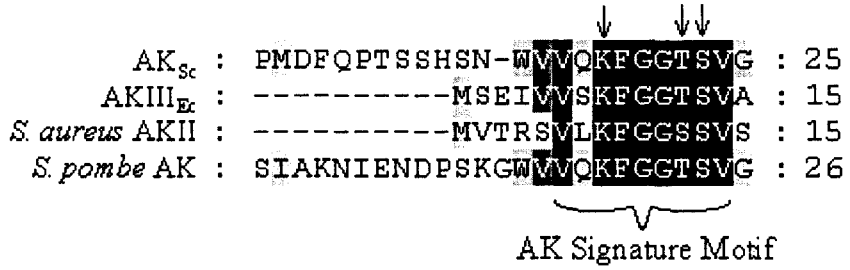

Figure 2.2. Amino acid alignment of the N-terminal portion of AKs showing the AK Signature Motif, arrows point to K18, T22, and S23. SwissProt accession numbers: AK_{Sc} (P10869); $AKIII_{Ec}$ (NP_418448); *Staphylococcus aureus* AKII (AAG42244), *S. aureus* AKII; *Schizosaccharomyces pombe* AK (CAA18652), *S. pombe* AK. For the sake of clarity, only 4 sequences are shown. The original alignment included 28 sequences of microbial and plant origin.

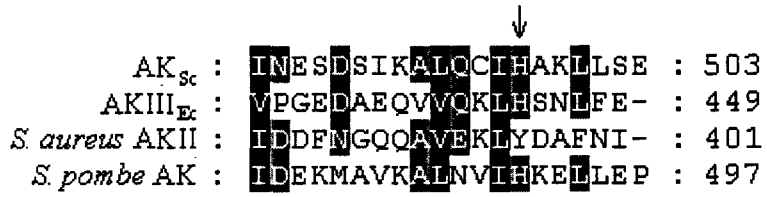

Figure 2.3. Amino acid alignment of AKs showing the conserved AK_{Sc}-H497, indicated by the arrow.

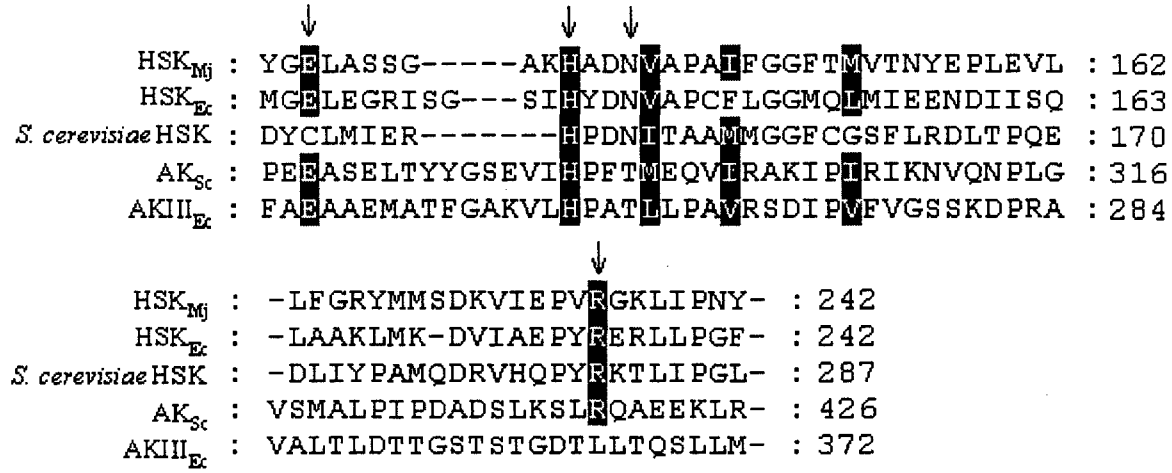

Figure 2.4. Alignment of HSKs and AKs showing the HSK_{Mj} active site residues E130, H138, N141, and R235, indicated by arrows. SwissProt accession numbers: HSK_{Mj} (G64437), HSK_{Ec} (NP_414544), *S. cerevisiae* HSK (NP_011890).

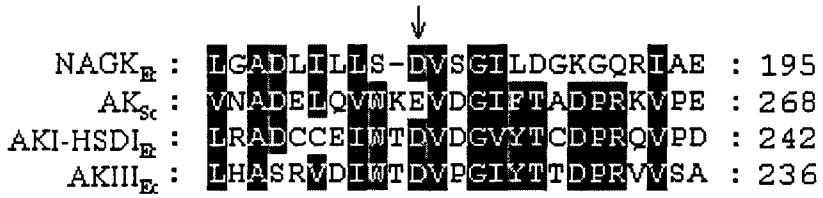

Figure 2.5. Alignment of NAGK$_{Ec}$, AK$_{Sc}$, AKI-HSDI$_{Ec}$, and AKIII$_{Ec}$. SwissProt accession numbers: NAGK$_{Ec}$ (P11445), AK$_{Sc}$ (KIBYD), AKI-HSDI$_{Ec}$ (NP_414543), AKIII$_{Ec}$ (NP_418448).

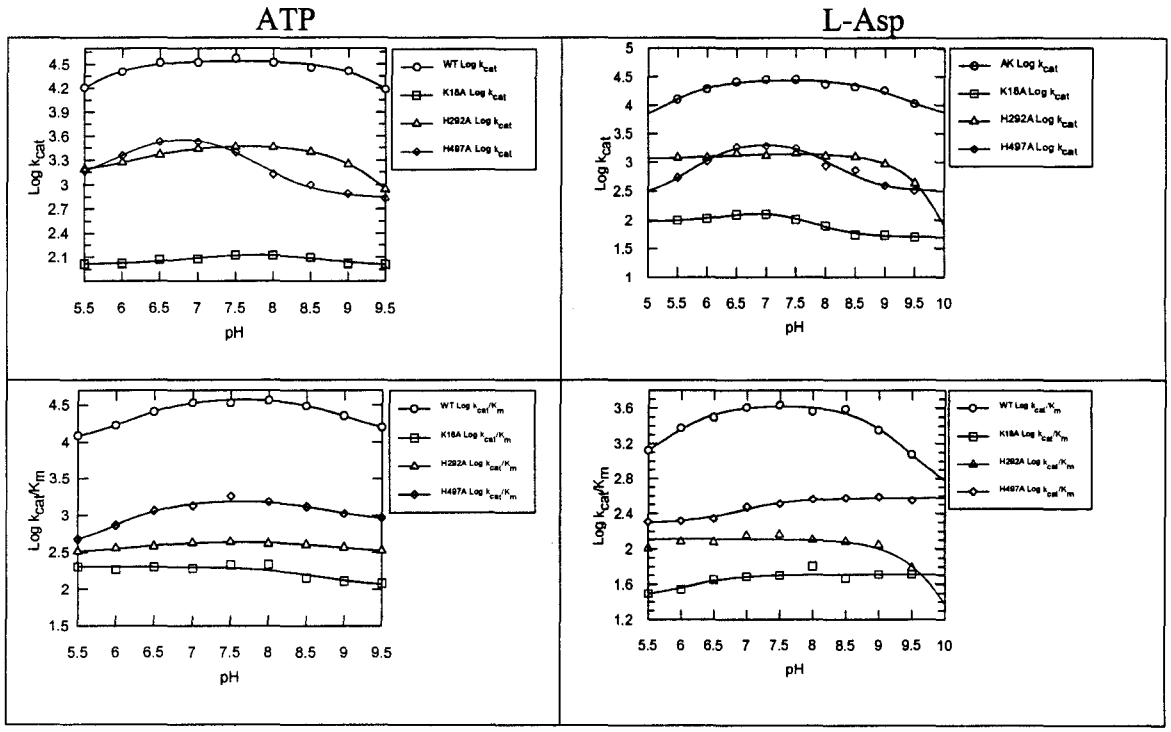

Figure 2.6. Effect of pH on AK_{Sc}, K18A, H292A, and H497A k_{cat} and k_{cat}/K_m. Data were fit to eq (3), (4), and (5) as indicated in Material and Methods and the associated pKas are summarized in Table 2.5.

Chapter 3

Small Molecule Functional Discrimination of the Kinases Required for the Microbial Synthesis of Threonine and Isoleucine

David Bareich, Kalinka Koteva, Ishac Nazi, Gerard D. Wright

3.1. Preface

The experimental work reported in this paper was obtained by a collaboration of three members in the laboratory of Dr. G. Wright. Ishac Nazi was responsible for the cloning of *Schizosaccharomyces pombe* homoserine kinase. Dr. Kalinka Koteva was responsible for the synthesis of the four bisubstrate compounds outlined in Scheme 3.2. The paper has been submitted to Bioorganic and Medicinal Chemistry, but, at the time of this writing, has not been accepted.

3.2. Abstract

The biosynthesis of L-threonine and L-isoleucine in bacteria and in fungi requires the action of 2 amino acid kinases: aspartate kinase and homoserine kinase. Although these kinases bind similar substrates and catalyze analogous phosphotransfer chemistry, they do not show high amino acid sequence homology. We show that despite this difference, both kinases form a ternary complex consisting of enzyme – adenosine triphosphate – amino acid to accomplish phosphoryl transfer. With this similarity in mind, we set out to identify molecules that could lead to inhibitors with activity against both kinases in the pathway. We synthesized a series of aspartic acid – adenosine bisubstrate compounds separated by a variable length alkyl linker that we hypothesized could bind to these kinases. These bisubstrate compounds only inhibited the bacterial aspartate kinase. These results reveal unexpected differences in small molecule interactions among these functionally similar enzymes.

3.3. Introduction

Fungi and bacteria can biosynthesize L-threonine and L-isoleucine using a metabolic pathway which includes two kinases: aspartate kinase (AK) and homoserine kinase (HSK). There is good evidence that this pathway is suitable for target-based antibiotic design, particularly because the pathway is not found in mammals (Viola 2001). These kinases discriminate amino acid substrates that differ only in the oxidation state of a single carbon, albeit with significantly different chemical properties, Scheme 3.1.

AK is the first step in the aspartate pathway, which in fungi is responsible for the production of threonine, isoleucine and methionine and in bacteria lysine is an additional product of the pathway (Viola 2001). AKs catalyze the transfer of the γ-phosphate of adenosine triphosphate, (ATP) to L-Asp producing adenosine diphosphate (ADP) and 4-phosphoaspartate, Scheme 3.1. The 4-phosphoaspartate product is unstable and has been shown to have a half life of approximately 1 h at pH 8.0 (Angeles et al. 1992). The yeast *Saccharomyces cerevisiae* has a single AK, (AK_{Sc}), which is regulated by feedback inhibition by L-threonine, whereas the bacterium *Escherichia coli* has three AKs which are separately regulated, two of which are bifunctional with homoserine dehydrogenase, regulated by L-threonine (AKI-$HSDI_{Ec}$) and L-methionine (AKII-$HSDII_{Ec}$), and the third is monofunctional, ($AKIII_{Ec}$), and is regulated by L-lysine (Stadtman et al. 1961; Ramos et al. 1991). AKI-$HSDI_{Ec}$ and $AKIII_{Ec}$ have been shown to catalyze phosphotransfer to a

structurally diverse set of L-Asp analogs (Angeles et al. 1992; Keng and Viola 1996). Lastly, studies of the kinetic mechanism of AK have shown that ATP and L-Asp bind to AKI-HSDI$_{Ec}$ and AKIII$_{Ec}$ in a random manner (Shaw and Smith 1977; Angeles and Viola 1990).

HSKs catalyze the first committed step towards the biosynthesis of threonine and isoleucine. The reaction they catalyze is similar to that catalyzed by AKs with the difference that the phosphoryl acceptor on L-homoserine is an alcohol rather than a carboxylic acid, Scheme 3.1. This distinction results in a significant difference in the products, *O*-phosphohomoserine is thermodynamically stable whereas 4-phosphoaspartate is not. *E. coli* homoserine kinase, (HSK$_{Ec}$), has been shown to accept a wide range of substrates and inhibitors similar to AKI-HSDI$_{Ec}$ and AKIII$_{Ec}$ (Huo and Viola 1996). Substrates bind to HSK$_{Ec}$ in a preferred order random mechanism where ATP is preferentially bound before L-homoserine (Shames and Wedler 1984). Crystal structures of *Methanococcus jannaschii* homoserine kinase, (HSK$_{Mj}$), with ADP, (Zhou et al. 2000) as well as with homoserine (Krishna et al. 2001) have been solved.

The similarity between the yeast enzymes AK$_{Sc}$ and HSK$_{Sc}$ at the primary amino acid sequence level is limited to 15% similarity and only 5% identity. Despite this lack of primary sequence similarity, these kinases catalyze phosphoryl transfer reactions between very similar substrates and our results show they use similar mechanisms. We hypothesized that compounds could be designed to inhibit both kinases using their similar

substrates as templates. Such compounds could serve as leads to new antibiotics and the ability to inhibit two enzymes could reduce the risk of development of antibiotic resistance.

3.4. Results and Discussion

3.4.1. Steady State Characterization of the Aspartate Pathway Kinases

The steady state kinetic parameters for ATP and the amino acid for each kinase were determined, Table 3.1. The steady state parameters for AK_{Sc} with and without the N terminal hexa-histidine tag were identical, (data not shown). The steady state parameters determined for HSK_{Sp} were in agreement with previously published values for the orthologous enzyme from *E. coli* (Theze et al. 1974; Burr et al. 1976). The $AKIII_{Ec}$ L-Asp K_m was 2 fold higher than the yeast enzyme but was 10 times higher than in a previous report, (Keng and Viola 1996) which may be due to the presence of the N terminal hexa-histidine tag. The absence of reported ATP K_m values for any AKIII prevents comparison of the value determined here.

3.4.2. AK and HSK Have Similar Mechanisms of Phosphoryl Transfer

The kinases of the aspartate pathway could transfer the γ-phosphate of ATP to the amino acid in one of two ways. A direct displacement mechanism would see the γ-phosphate of ATP transferred directly to the amino acid. In a double displacement mechanism, the γ-phosphate would be attacked by an enzyme nucleophile, creating a phosphoenzyme intermediate that may not be stable. This phosphoenzyme intermediate would then be attacked by the amino acid producing the phosphorylated amino acid.

A ping pong steady state kinetic mechanism is evidence for a double displacement mechanism and can be easily distinguished in initial velocity experiments as parallel lines on a double reciprocal plot. Initial velocity experiments were performed for each of the kinases and in all cases an intersecting pattern was observed, Figure 3.1, showing that the enzymes use a ternary complex mechanism and not a ping pong mechanism consistent with literature precedent for $AKIII_{Ec}$ (Shaw and Smith 1977) and *E. coli* HSK (Shames and Wedler 1984). However, a ternary complex mechanism does not rule out the possibility that the enzymes use a double displacement mechanism.

To assess the order of substrate binding in AK_{Sc}, the patterns of ADP product inhibition were determined against both substrates, Figure 3.2. Product inhibition studies with 4-phosphoaspartate are not possible due to its instability. The mixed pattern of

inhibition observed in the ADP product inhibition studies, Figure 3.2, suggests that L-Asp and ATP are bound in a random order, which agrees with the order of binding established for $AKIII_{Ec}$ (Shaw and Smith 1977) and the AK domain of $AKI\text{-}HSDI_{Ec}$ (Angeles and Viola 1990). Although the order of substrate binding has not been established for HSK_{Sp}, it has for *S. cerevisiae* HSK which uses a preferred random order (Shames and Wedler 1984). Thus, it is likely that the three kinases in this study bind substrates in a similar order.

To determine if a stable phosphoenzyme intermediate exists, we incubated each of the kinases with γ-^{32}P-ATP, separated them on an SDS polyacrylamide gel, and used autoradiography to determine if the protein bands were labelled with ^{32}P, Figure 3.3. No radioactivity was detected for any of the kinase bands, however, the positive control, VanS from *E. faecium* which is autophosphorylated on a histidine, (Wright et al. 1993) was labelled with ^{32}P. This finding indicates either that no phosphoenzyme intermediate exists or that it is kinetically or thermodynamically unstable.

If the kinases are forming a labile phosphoenzyme intermediate, they would show ATPase activity and produce ADP and inorganic phosphate in the absence of the amino acid. We assessed the stability of ATP when incubated with each of the kinases in an ATPase assay. Using thin layer chromatography, the ^{32}P in ATP, inorganic phosphate, and *O*-phosphohomoserine or 4-phosphoaspartate were visualized following incubation of the kinases with γ-^{32}P-ATP, and the ^{32}P in ADP was visualized following incubation of

the kinases with α-^{32}P-ATP (data not shown). The results for AK_{Sc}, $AKIII_{Ec}$, and HSK_{Sp} are similar in all respects. Control incubations of the labelled ATP with kinase inactivated by boiling established the intensity, location, and size of the ATP spot. Incubations of the kinases with either γ-^{32}P-ATP or α-^{32}P-ATP appeared the same as the controls, indicating the kinases do not possess ATPase activity. Identical incubations supplemented with the appropriate amino acid showed that the kinases were active. This is shown in the α-^{32}P-ATP incubations as an increase in the ADP spot, whereas the γ-^{32}P-ATP incubations show the presence of *O*-phosphohomoserine for HSK_{Sp} and a smear resulting from the decay of the unstable 4-phosphoaspartate for both AK_{Sc} and $AKIII_{Ec}$.

Taken together, the lack of a ping pong mechanism, lack of ^{32}P labelling of the kinases, and lack of ATPase activity leaves a direct displacement mechanism as the most likely means of phosphoryl transfer in each of the kinases of the aspartate pathway. Therefore, despite low levels of amino acid homology, AK and HSK share aspects of steady state mechanism.

3.4.3. Small Molecule Inhibitors of AK_{Sc}, $AKIII_{Ec}$, and HSK_{Sp}

A summary of the AK_{Sc}, $AKIII_{Ec}$, and HSK_{Sp} IC_{50} values for the *in vivo* allosteric inhibitors L-Thr (AK_{Sc}) and L-Lys ($AKIII_{Ec}$), aspartate pathway substrates L-homoserine and L-Asp, and amino acid analogs hydroxynorvaline and L-norvaline are shown in Table 3.2. The IC_{50} values for the *in vivo* allosteric inhibitors were within 3 fold of

previously published values (Ramos et al. 1991; Ogawa-Miyata et al. 2001). The AK_{Sc} IC_{50} value for hydroxynorvaline compares favourably to the previously reported values at fixed concentrations of hydroxynorvaline (Ramos et al. 1991; Ramos and Calderon 1992). The lack of inhibition of $AKIII_{Ec}$ by hydroxynorvaline may reflect differential binding of small molecules by the two AKs.

3.4.4. Bisubstrate Compound Inhibition of the Aspartate Pathway Kinases

The kinases of the aspartate pathway bind substrates that differ only in the oxidation state of a single carbon on the amino acid, Scheme 3.1, and use a common phosphotransfer mechanism. These similarities led us to hypothesize that inhibitors, using the substrates as templates, could be designed to inhibit both kinases. These could be useful as leads in the design of compounds that would show activity against two enzymes in the same metabolic pathway, and such compounds could be potent antifungal agents with decreased potential to select for target-based resistance. We synthesized a series of aspartate-adenosine bisubstrate compounds linked through the phosphate donor and acceptor sites with a variable length alkyl linker, Scheme 3.2. One or more of these compounds could be bound by the kinases causing competitive inhibition against both substrates.

The bisubstrate compounds were poor inhibitors of AK_{Sc} and HSK_{Sp}, Table 3.3, but good inhibitors of $AKIII_{Ec}$ with the longest bisubstrate compound **8** giving an IC_{50}

value of 75 μM. Furthermore, an interesting trend is evident in the IC_{50} values for the bisubstrate compounds against $AKIII_{Ec}$: inhibition increases with linker length. This may be giving an indication of the distance between the two substrate or product binding sites in the enzyme.

The inhibition parameters for the two longest bisubstrate compounds **7** and **8** were assessed against $AKIII_{Ec}$, Table 3.4. Surprisingly, these compounds did not show competitive inhibition against either substrate. For both compounds the mechanism of inhibition was mixed with respect to ATP, however, noncompetitive and uncompetitive gave the best fits for compounds **7** and **8** versus L-Asp, respectively. The uncompetitive and noncompetitive mechanisms of inhibition suggest that the L-Asp portion of the bisubstrate compounds is not solely binding to the L-Asp binding pocket in $AKIII_{Ec}$. A similar situation was observed in crystal structures of thymidylate kinase and the bisubstrate inhibitor Ap_5T which clearly show the ADP portion of the bisubstrate compound in a cavity on the surface of the enzyme (Haouz et al. 2003). The low μM K_i values for these bisubstrate compounds places them among the best known inhibitors of any AK.

The observation that the bisubstrate compounds are good inhibitors of $AKIII_{Ec}$ and poor inhibitors of AK_{Sc} was unexpected. Considering our results showed that these AKs use the same phosphotransfer mechanism and bind identical substrates, although they are only 15% similar as assessed by sequence alignment of the two primary amino

acid sequences, we predicted the bisubstrate compounds to inhibit both AKs. However, AK_{Sc} and $AKIII_{Ec}$ do respond unequally to L-Thr and hydroxynorvaline, Table 3.2, perhaps due to differences in their ACT domains which are responsible for their allosteric response to L-Thr and L-Lys, respectively (Aravind and Koonin 1999).

The lack of inhibition of HSK_{Sp} may be explained by selection against a substrate analog that resembles L-Asp rather than homoserine. The crystal structure of HSK_{Mj} complexed with homoserine and an ATP analog reveal that HSK_{Mj}-N141, which is equivalent to HSK_{Sp}-N135, interacts with the δ-OH of homoserine (Krishna et al. 2001). This side chain may be responsible for selecting against binding of L-Asp and for that matter our bisubstrate compounds.

3.5. Conclusions

We have shown that AK_{Sc}, $AKIII_{Ec}$, and HSK_{Sp} use similar mechanisms in transferring the γ-phosphate from ATP to their cognate amino acid substrate, however this similarity and the fact that the substrates only differ in the oxidation state of a single carbon, does not translate into comparable ability to bind a series of new bisubstrate compounds. The lack of inhibition of HSK_{Sp} by the bisubstrate compounds in this study may reflect a requirement for sp^2 vs. sp^3 hybridization at C4 of the amino acid position. The differential inhibition of AK_{Sc} and $AKIII_{Ec}$ by the bisubstrate analogues on the other

hand points to an unexpected difference in small molecule recognition among the members of these metabolic enzymes. This suggests therefore, that design of inhibitors of AK may not result in broad spectrum antimicrobial activity.

3.6. Acknowledgements

The authors wish to thank Dr. Xiao Dong Wang for supplying *E. faecium* VanS, the McMaster MOBIX DNA sequencing lab, McMaster Department of Chemistry NMR and mass spectrum labs. This research was supported by the Ontario Research and Development Fund and Crompton Corporation. G.D.W. is supported by a Canada Research Chair in Antibiotic Biochemistry.

3.7. Experimental

3.7.1. AK_{Sc} cloning, expression, and purification

Cloning of the *HOM3* gene, which encodes AK_{Sc}, has been previously reported (Bareich et al. 2003a). Two 1 L LB + 50 μg/mL kanamycin overnight cultures of *E. coli* BL21(DE3)/pET28 + AK_{Sc} were used to inoculate 28 L of LB supplemented with 50 μg/mL kanamycin. The culture was grown at 37 °C, with 300 rpm agitation and a sparge rate of 40 L air/min, to an OD_{600} of approximately 0.7. The temperature was reduced to 20 °C and the culture was induced with 1 mM IPTG for 4 hours. The harvested cells

were resuspended in a minimal volume of 20 mM 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES) pH 8.0 and lysed with 3 passes through a French Pressure cell at 10,000 psi in the presence of 1 mM phenylmethylsulfonyl fluoride (PMSF). The cell debris was pelleted at 10,000 x g for 10 min, the supernatant was applied to a 40 mL Q Sepharose column and the column was washed with 20 mM HEPES pH 8.0 until the $OD_{280\,nm}$ decreased to less than 100 mAu. AK_{Sc} was eluted with a linear gradient of 0 – 400 mM NaCl in 20 mM HEPES pH 8.0. Active fractions were pooled and applied to an 8 mL Ni-NTA Agarose column, the column was washed with 20 mM HEPES pH 8.0 until $OD_{280\,nm}$ decreased to less than 100 mAu. AK_{Sc} was eluted with a gradient of imidazole from 0 to 200 mM in 20 mM HEPES pH 8.0. Active fractions were dialyzed overnight at 4 °C against 4 L of 20 mM HEPES pH 8.0. The 0.05 mg/mL AK_{Sc} (total yield was 8.5 mg) was frozen with 10% glycerol at –80 °C.

3.7.2. $AKIII_{Ec}$ cloning, expression, and purification

$AKIII_{Ec}$ was cloned using the PCR primers $AKIII_{Ec}$-1 and $AKIII_{Ec}$-2, Table 3.5. The PCR reaction consisted of 50 pg *E. coli* GM48 genomic DNA, 20 pmol of each primer, 20 nmol dNTP's, 20 mM KCl, 20 mM Tris-HCl (pH 8.8), 10 mM $(NH_4)_2SO_4$, 0.1 % Triton X-100, 120 μmol $MgSO_4$, 2 U Taq DNA polymerase in a final volume of 20 μL. Taq DNA polymerase was the final component added to the reactions at 95 °C, the reactions were given 30 cycles of: 95 °C – 1 min, 50 °C – 1 min, 72 °C – 1.5 min.

The PCR amplified $AKIII_{Ec}$ gene was cloned into pET28 using standard techniques to create the sequence confirmed construct pET28 + $AKIII_{Ec}$. This construct was transformed into *E. coli* BL21(DE3) cells, allowing expression of $AKIII_{Ec}$ with a hexa-histidine tag fused to its N terminus.

Three 1 L cultures of LB supplemented with 50 μg/mL kanamycin were inoculated with *E.* coli BL21(DE3)/pET28 + $AKIII_{Ec}$ and grown at 37 °C to an OD_{600} of 0.6. The three flasks were cooled in an ice-water bath for 5 min to reduce the temperature to approximately 14 °C, IPTG was added to 1 mM and the cells were incubated overnight in a 14 °C shaker. The harvested cells were resuspended in a minimal volume of 20 mM HEPES pH 7.0 and lysed with three passes through a French Pressure cell at 10,000 psi, in the presence of 1 mM PMSF and 0.1 mM DTT. Cell debris was pelleted at 10,000 x g for 10 min and the supernatant was applied to a 9 mL freshly poured Q Sepharose column. Protein was eluted with a linear gradient of 0 to 500 mM NaCl in 20 mM HEPES pH 7.0. The majority of eluted fractions had $AKIII_{Ec}$ activity which was pooled and 10% was loaded onto an 8 mL Ni-NTA Agarose column. $AKIII_{Ec}$ was eluted with a linear gradient of 25 to 150 mM imidazole in 20 mM HEPES 7.0. Active fractions were pooled and dialyzed overnight at 4 °C in 4 L of 20 mM HEPES pH 7.0. 43 mg of $AKIII_{Ec}$ was frozen with 10% glycerol at –80 °C at 1.95 mg/mL and was stable for several months.

3.7.3. *Schizosaccharomyces pombe* HSK cloning, expression, and purification

Schizosaccharomyces pombe HSK, (HSK_{Sp}), was cloned using the PCR primers HSK_{Sp}-1 and HSK_{Sp}-2, Table 3.5. The PCR reaction consisted of 50 pg *S. pombe* genomic DNA, 40 pmol of each primer, 25 nmol dNTP's, 20 mM KCl, 20 mM Tris-HCl (pH 8.8), 10 mM $(NH_4)_2SO_4$, 0.1 % Triton X-100, 50 μmol $MgSO_4$, 1 U Vent DNA polymerase in a final volume of 50 μL. Vent DNA polymerase was the final component added to the reaction at 94 °C, the reaction underwent 25 cycles of: 94 °C – 1 min, 56 °C – 1 min, 72 °C – 1 min.

The PCR amplified HSK_{Sp} gene was cloned into the *Nhe* I and *Hind* III sites of pET28 using standard techniques to create the sequence confirmed construct pET28 + HSK_{Sp}. This construct was transformed into *E. coli* BL21(DE3) cells, allowing expression of HSK_{Sp} with a hexa-histidine tag fused to its N terminus.

Three 1 L LB + 50 μg/mL cultures of *E.* coli BL21(DE3)/pET28 + HSK_{Sp} were grown at 37 °C to an OD_{600} of 0.6. The three flasks were cooled in an ice-water bath for 5 min to reduce the temperature to approximately 14 °C, 1 mM IPTG was added and the cells were incubated overnight in a 14 °C shaker. The harvested cells were resuspended in a minimal volume of 20 mM HEPES pH 8.0 and lysed with three passes through a French Pressure cell at 10,000 psi, in the presence of 1 mM PMSF. Cell debris was

pelleted at 10,000 x g for 10 min and the supernatant was applied to an 8 mL Ni-NTA Agarose column. Protein was eluted with a gradient of imidazole from 0 to 200 mM in 20 mM HEPES pH 8.0. Active fractions were dialyzed overnight at 4 °C against 4 L of 20 mM HEPES pH 8.0. The 5.7 mg/mL HSK_{Sp} (total yield was 154 mg) was frozen with 10% glycerol at –80 °C.

3.7.4. Enzyme Assays

AK and HSK activities were monitored indirectly by coupling ADP production to oxidation of NADH using pyruvate kinase and lactate dehydrogenase at 30°C. NADH oxidation was monitored kinetically at 340 nm using a Molecular Devices Spectramax microtitre platereader for a minimum of 10 min. Activity assays for identification of active fractions during purification were initiated with the appropriate amino acid (HSK_{Sp}, [L-Hse]=35 mM; AK_{Sc}, [L-Asp]=25 mM; $AKIII_{Ec}$ [L-Asp]=32 mM) at 5 mM ATP. Inhibition assays (K_i and IC_{50}) were set up to allow a 20 min pre-incubation at 30 °C of enzyme, buffer, salts, and inhibitor, followed by addition of pyruvate kinase, lactate dehydrogenase, phosphoenol pyruvate, and NADH. Reactions were initiated with the appropriate amino acid (HSK_{Sp}, [L-Hse]=10 mM, AK_{Sc}, [L-Asp]=32 mM; $AKIII_{Ec}$, [L-Asp]=35 mM) at 5 mM ATP. All assays were composed of 100 mM HEPES pH 7.5, 40 mM $MgCl_2$, 10 mM KCl, 0.33 U pyruvate kinase and 0.66 U lactate dehydrogenase, 2.5 mM phosphoenol pyruvate, 1 mM NADH in a 100 μL final volume.

3.7.5. Product Inhibition Studies for AK_{Sc}

Product inhibition studies were performed on AK_{Sc} to assess the order of substrate binding. It is not possible to use 4-phosphoaspartate in such studies due to its instability. Product inhibition studies with ADP are not possible using the pyruvate kinase – lactate dehydrogenase assay since pyruvate kinase uses ADP as a substrate. To avoid this, AK_{Sc} was monitored by coupling 4-phosphoaspartate production to NADPH oxidation using aspartate semialdehyde dehydrogenase, ASD_{Sc}. Cloning, expression and purification of ASD_{Sc} have been previously described (Bareich et al. 2003b). Assays were performed at 30 °C using an amount of ASD_{Sc} that had been determined not to be rate limiting at the highest possible AK_{Sc} rates. Buffer and metals were added at 100 mM HEPES pH 7.5, 40 mM $MgCl_2$, 10 mM KCl. The oxidation of 0.8 mM NADPH in a total assay volume of 100 μL was followed at 340 nm in a Molecular Devices Spectramax microtitre platereader, reactions were initiated with L-Asp in all cases.

3.7.6. Data Fitting

All data fitting performed used GraFit version 4 software (Leatherbarrow 2001). Michaelis-Menten steady state kinetic parameters were determined by fitting the data to eq (1). IC_{50} values were obtained by fitting the data to the four parameter eq (2), where A = minimum response plateau, D = maximum response plateau, I = concentration of inhibitor, S = slope factor. K_i values were determined from global fits of the data to all

inhibition equations, the best fit was assessed visually and with F-tests. The inhibition equations used were: competitive (3), non-competitive (4), mixed (5), uncompetitive (6).

$$\upsilon = \frac{V_{\max} S}{(K_m + S)} \tag{1}$$

$$y = \frac{A - D}{1 + \left(\frac{I}{IC_{50}}\right)^S} + D \tag{2}$$

$$\upsilon = \frac{V \max S}{\left(K_m\left(1 + \frac{I}{K_i}\right) + S\right)} \tag{3}$$

$$\upsilon = \frac{V_{\max} S}{\left(K_m\left(1 + \frac{I}{K_i}\right) + S\left(1 + \frac{I}{K_i}\right)\right)} \tag{4}$$

$$\upsilon = \frac{V_{\max} S}{\left(K_m\left(1 + \frac{I}{K_i}\right) + S\left(1 + \frac{I}{K'_i}\right)\right)} \tag{5}$$

$$\upsilon = \frac{V_{max} S}{\left(K_m + S\left(1 + \frac{I}{K_i} \right) \right)} \qquad (6)$$

3.7.7. Detection of a Stable Phosphoenzyme Intermediate

The presence of a stable phosphoenzyme intermediate in AK_{Sc}, $AKIII_{Ec}$, and HSK_{Sp} was assessed following 20 min room temperature incubations of 5 μg of each kinase and the positive control VanS from *Enterococcus faecium* (Wright et al. 1993). The incubations included 100 mM HEPES pH 7.5, 40 mM $MgCl_2$, 10 mM KCl, 0.5 mM ATP with γ-^{32}P-ATP (2.2 x 10^6 dpm). After adding an equal volume of sodium dodecylsulfate (SDS) polyacrylamide gel loading buffer which contained 10% SDS, 100 mM Tris pH 8.8, 2 mM EDTA, 10% glycerol, 0.5 mM 1,4-dithio-D,L-threitol, 0.3 mM bromophenol blue, the reactions were chromatographed on an 11% SDS polyacrylamide gel without boiling and stopped before the bromophenol blue dye front ran off the end of the gel. The ^{32}P was detected by autoradiography and the gel was stained with Coomassie stain.

3.7.8. ATPase Assay

The potential presence of an unstable phosphoenzyme intermediate in AK_{Sc}, $AKIII_{Ec}$, and HSK_{Sp} was assessed following a 30 min room temperature incubation that was initiated with enzyme. All reactions were buffered with 100 mM HEPES pH 7.5, 40 mM $MgCl_2$, 10 mM KCl in a final volume of 50 µL. The concentration of ATP spiked with approximately 2.2 x 10^6 dpm of either α-^{32}P-ATP or γ-^{32}P-ATP in AK_{Sc}, $AKIII_{Ec}$, and HSK_{Sp} reactions was 2.5, 5, and 1.5 mM, respectively. L-Asp was added to AK_{Sc} reactions at 8 and 13 mM and to $AKIII_{Ec}$ reactions at 7.2 and 11 mM. L-Homoserine was added to HSK_{Sp} reactions at 1 and 1.5 mM. Reactions were stopped by the addition of 5 µL of 500 mM ethylenediaminetetraacetic acid (EDTA) and 3 µL samples were spotted at the origin on 20 x 20 cm Silica gel 60 thin layer chromatography plates. The samples were chromatographed using a mobile phase of 3:1:6 dioxane : ammonium hydroxide : water adapted from (Sillero et al. 2001).

3.7.9. Preparation of compounds 1 - 4

The Boc-Asp-OBzl cesium salt was prepared by adding 4 mL H_2O to a 20 mL methanol solution of Boc-Asp-OBzl (2 g). The solution was neutralized with a 20% solution of cesium carbonate in water (approximately 15 mL) and after evaporation of the methanol and lyophyilization of H_2O the cesium salt was obtain as crystals.

General strategy for compounds **1 - 4**: the appropriate dibromoalkane (2.6eq) was added to a stirred solution of Boc-Asp-OBzl cesium salt (1 eq) in 10 ml DMF at room temperature and stirred overnight (Bodansky and A. 1984). The reaction was diluted with 100ml of water and the product extracted with ethylacetate (3 x 60 ml). The crude compounds were purified by column chromatography (Silica gel G60 A) using chloroform as the mobile phase. Fractions were analyzed by thin layer chromatography on Silica gel 60 plates visualized by UV and ninhydrin spray.

Compounds **1-4**: (**1**) ^{1}H NMR ($CDCl_3$) 7.33 (s, 5H), 5.45 (m,1H), 5.18 (s,2H), 3.37-3.43 (m,4H), 2.98 (m,2H), 1.46 (m,4H), 1.43 (s, 9H), 1.21-1.28 (t, 2H, $J_1$7.1Hz, J_2 7.2Hz). ES-MS m/z (relative intensity) 474 (M+2, 50 %), 494.3 (M+Na, 100%). R_f0.92 ($CHCl_3$:MeOH:AcOH=95:5:3). (**2**) ^{1}H NMR ($CDCl_3$) 7.33 (s, 5H), 5.45 (m,1H), 5.18 (s,2H), 3.37-3.44 (t,4H, $J_1$6.7Hz, J_2 6.7Hz), 2.98 (m,2H), 1.80-1.89 (m,4H), 1.43 (s, 9H), 1.44-1.50 (m, 4H). ES-MS m/z (relative intensity) 488 (M+2, 90 %), 508.1 (M+Na, 10%). R_f0.98 ($CHCl_3$:MeOH:AcOH=95:5:3). (**3**) ^{1}H NMR ($CDCl_3$) 7.33 (s, 5H), 5.45 (m,1H), 5.18 (s,2H), 3.37-3.44 (t,4H, $J_1$6.7Hz, J_2 6.7Hz), 2.98 (m,2H), 1.79-1.93 (m,4H), 1.34-1.51 (s, 17H). ES-MS m/z (relative intensity) 502 (M+2, 30%), 524 (M+Na, 40 %). R_f 0.8 (EtOAc:Hexanes=1:1). (**4**) ^{1}H NMR ($CDCl_3$) 7.33 (s, 5H), 5.45 (m,1H), 5.18 (s,2H), 3.37-3.44 (t,4H, $J_1$6.7Hz, J_2 6.7Hz), 2.98 (m,2H), 1.79-1.93 (m,4H), 1.34-1.51 (s, 19H). ES-MS m/z (relative intensity) 516 (M+2, 8%), 559 (M+K, 10%). R_f0.95 ($CHCl_3$:MeOH:AcOH=95:5:3).

3.7.10. Preparation of bisubstrate compounds 5 - 8 from 1 - 4

Separately compounds **1 - 4** (1 eq) were added to a mixture of N-benzoyl-2', 3'-O-isopropylideneadenosine **9** (1 eq), tetrabutylammoniumbromide (2 eq) and anhydrous DMSO (5 ml). KOH (2.5 eq, anhydrous, fine powder) was added and the mixture was vigorously stirred at room temperature under a nitrogen atmosphere for 2 h, quenched with cold water, and extracted with dichloromethane (3 x 30 ml) (Liu et al. 2000). The organic phase was dried over anhydrous sodium sulfate and concentrated under reduced pressure. The final product was purified by column chromatography, using chloroform as a mobile phase. The bisubstrate compounds **5 - 8**, were then obtained after Boc deprotection 1 hour at room temperature in 50% TFA/DCM, OBzl deprotection in 20 mL anhydrous methanol with 0.3 g potassium carbonate per g of unprotected bisubstrate compound stirred for 6 to 12 h at room temperature, and preparative TLC-purification.

Bisubstrate compounds **5 - 8**: (**5**) ^{1}H NMR ($CDCl_3$) 8.78 (s, 1H), 8.21 (s,1H), 5.97-5.99 (d,1H, J 4.5Hz), 5.10-5.13 (d,1H, J 6Hz), 3.37-3.43 (m,4H), 2.94 (m,2H), 1.46 (m,4H), 1.21-1.28 (t, 2H, $J_1$7.1Hz, J_2 7.2Hz). ES-MS m/z (relative intensity) 469 (M+1, 8%). R_f 0.59 ($CHCl_3$:MeOH=9:1). (**6**) ^{1}H NMR ($CDCl_3$) 8.78 (s, 1H), 8.21 (s,1H), 5.80 (d,1H, J 4.5Hz), 4.08-4.20 (m,4H), 3.37-3.55 (m,4H), 2.94 (m,2H), 1.44-1.50 (m,4H). ES-MS m/z (relative intensity) 480 (M-1, 35%). R_f0.42 ($CHCl_3$:MeOH=9:1). (**7**) ^{1}H NMR ($CDCl_3$) 8.78 (s, 1H), 8.21 (s,1H), 5.80 (d,1H, J 4.5Hz), 4.08-4.20 (m,4H), 3.33-3.55 (m,4H), 2.94 (m,2H), 1.39-1.58 (m,10H). ES-MS m/z (relative intensity) 497 (M+1,

8 %). R_f0.48 ($CHCl_3$:MeOH=9:1). (**8**) ^{1}H NMR ($CDCl_3$) 8.78 (s, 1H), 8.21 (s,1H), 5.80 (d,1H, J 4.5Hz), 4.08-4.20 (m,4H), 3.33-3.70 (m,4H), 2.94 (m,2H), 1.39-1.58 (m,12H). ES-MS m/z (relative intensity) 511 (M+1, 10 %). R_f0.54 ($CHCl_3$:MeOH=9:1).

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Table 3.1. Steady state kinetic parameters for AK_{Sc}, $AKIII_{Ec}$, HSK_{Sp}.

Enzyme	Varied Substrate	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($M^{-1}s^{-1}$)
AK_{Sc}	L-Asp	4.59 ± 0.51	46.6	10 200
$AKIII_{Ec}$	L-Asp	11.0 ± 1.6	43.4	3 950
HSK_{Sp}	L-Hse	0.52 ± .09	40.2	76 700
AK_{Sc}	ATP	0.94 ± 0.07	50.3	53 500
$AKIII_{Ec}$	ATP	1.08 ± .12	43.0	39 800
HSK_{Sp}	ATP	0.27 ± .06	40.8	149 000

Table 3.2. IC_{50} values for inhibitors of AK_{Sc}, $AKIII_{Ec}$, and HSK_{Sp}.

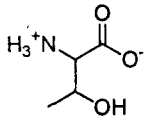
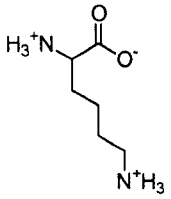
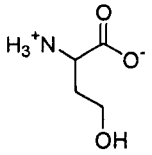
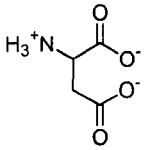
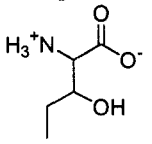
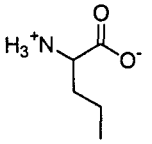
	AK_{Sc}	$AKIII_{Ec}$	HSK_{Sp}
 L-threonine	2.7 ± 0.87 mM	no inhibition up to 100 mM	no inhibition up to 100 mM
 L-lysine	no inhibition up to 120 mM	1.1 ± 0.076 mM	no inhibition up to 120 mM
 L-homoserine	no inhibition up to 10 mM	no inhibition up to 10 mM	substrate
 L-aspartic acid	substrate	substrate	no inhibition up to 10 mM
 hydroxynorvaline	6.2 ± 0.28 mM	no inhibition up to 50 mM	no inhibition up to 120 mM
 L-norvaline	74 ± 18 mM	61 ± 19 mM	no inhibition up to 120 mM

Table 3.3. IC_{50} values for the bisubstrate compounds. Values for bisubstrate compounds **5** and **6** are lower limits.

	5	**6**	**7**	**8**
AK_{Sc}	>0.5 mM	>0.5 mM	no inhibition	no inhibition
HSK_{Sp}	>0.5 mM	no inhibition	no inhibition	no inhibition
$AKIII_{Ec}$	>0.3 mM	>0.2 mM	0.23 ± 0.31 mM	0.075 ± 0.0056 mM

Table 3.4. Inhibition parameters for the bisubstrate compounds **7** and **8** against $AKIII_{Ec}$.

Bisubstrate Compound	Varied Substrate	Type of Inhibition	K_i (μM)	K_i' (μM)
7	ATP	Mixed	37 ± 3.7 μM	370 ± 100 μM
7	L-Asp	Non	130 ± 7.9 μM	
8	ATP	Mixed	11 ± 1.2 μM	120 ± 18 μM
8	L-Asp	Un	44 ± 2.4 μM	

Table 3.5. PCR primers used in cloning AK_{Sc}, $AKIII_{Ec}$, and HSK_{Sp}.

Primer	Sequence
AKIIIEc-1	5'-CCCGAATTCCATATGTCTGAAATTGTTGTCTCC
AKIIIEc-2	5'-CCCAAGCTTCTCGAGTTACTCAAACAAATTACTATGC
HSKSp-1	5'-GTCTCTAGAGCTAGCATGCAGAAATTTCAAATAAAAGTTCCAGC
HSKSp-2	5'-CGCGGATCCAAGCTTTCAAAAGTATTTGACTGTTGCACCGTC

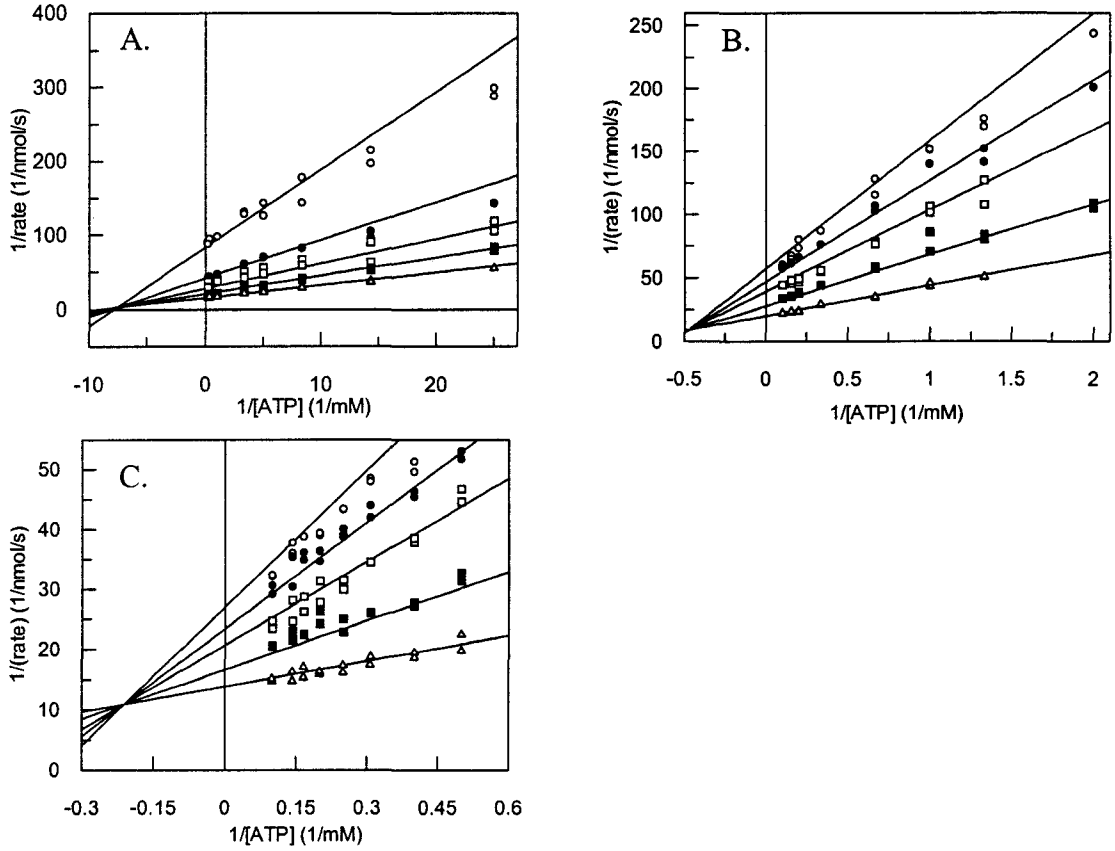


Figure 3.1. Kinases of the aspartate pathway follow a common ternary complex mechanism. Double reciprocal plots of A. HSK_{Sp} using homoserine concentrations of 0.1 (○), 0.25 (●), 0.5 (□), 1 (■), and 5 mM (△), B. $AKIII_{Ec}$ using L-Asp concentrations of 2.25 (○), 3 (●), 4 (□), 8 (■), and 25 mM (△), and C. AK_{Sc} using the same L-Asp concentrations as $AKIII_{Ec}$, are shown.

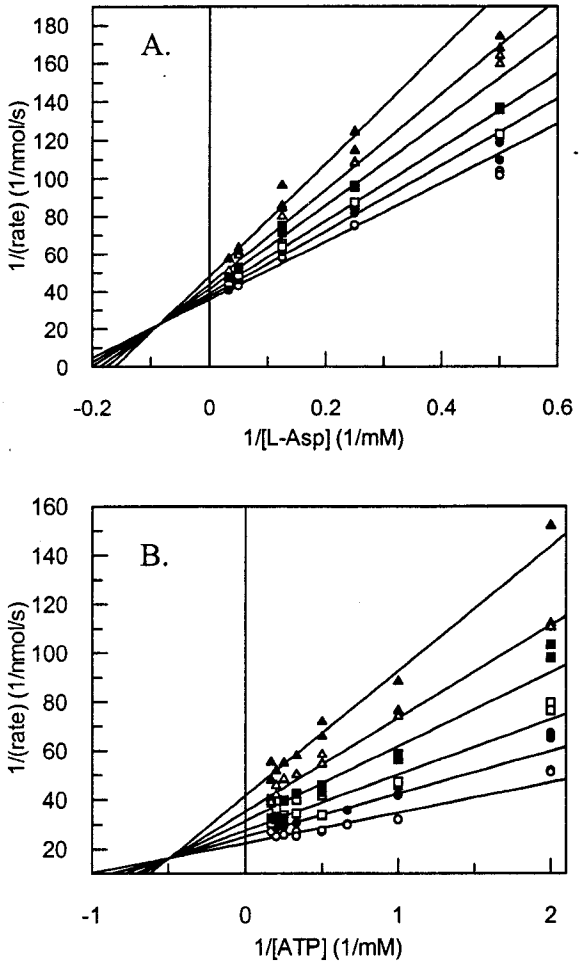


Figure 3.2. Product inhibition analysis of AK_{Sc} with respect to L-Asp, A., and ATP, B., using the AK-ASD assay. ADP concentrations are 0 (○), 0.2 (●), 0.4 (□), 0.7 (■), 1 (△), and 1.5 mM (▲).

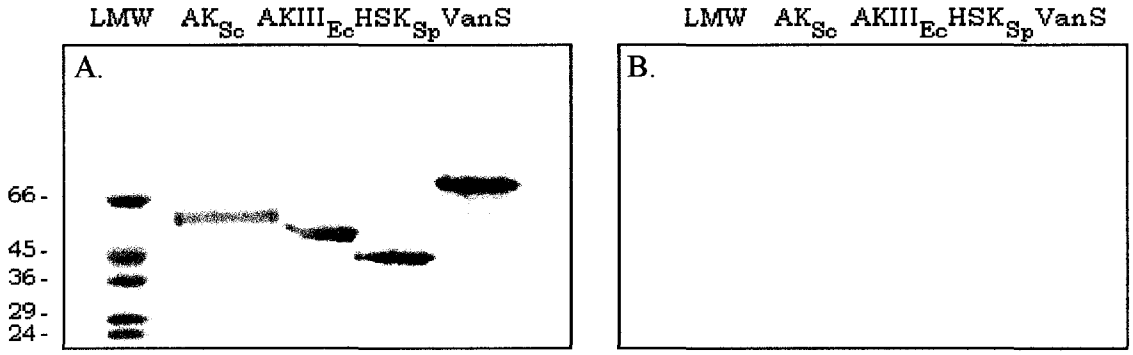
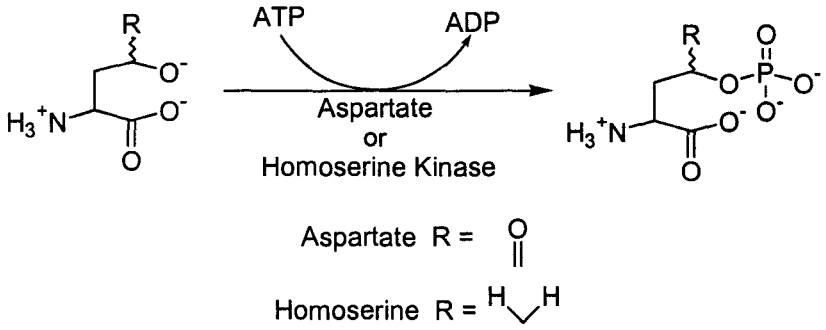
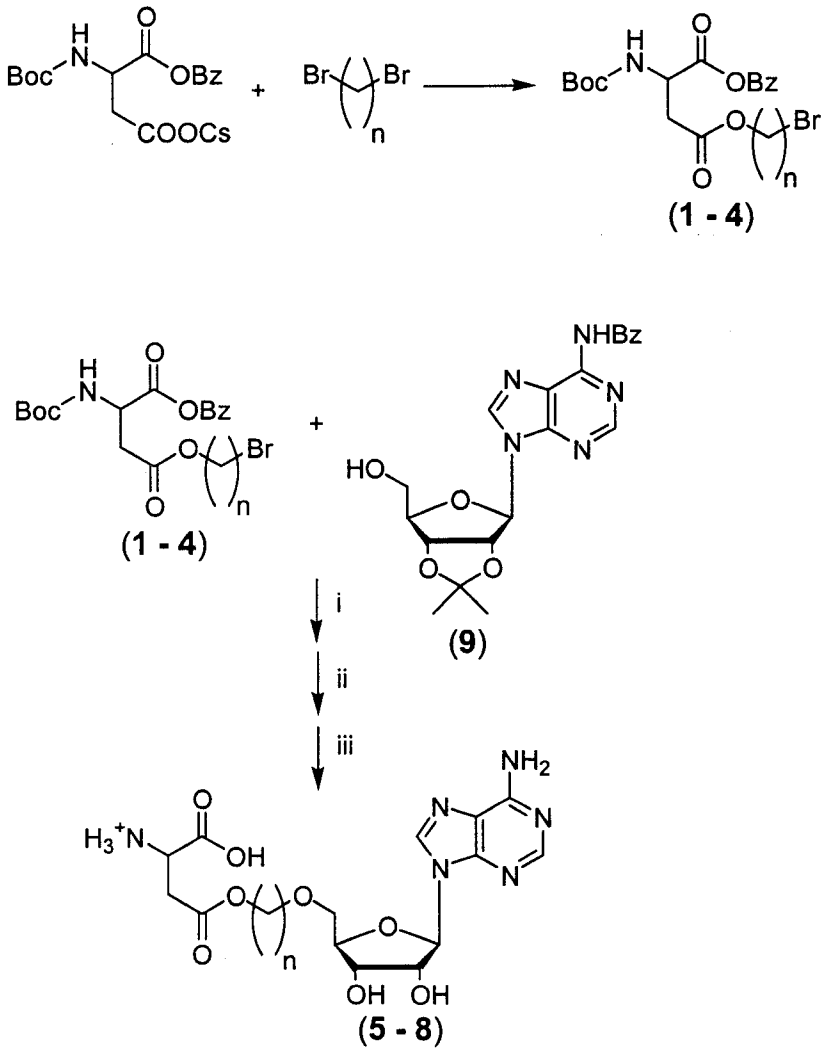


Figure 3.3. Aspartate pathway kinases are not phosphorylated by ATP. A. Coomassie stained gel of AK_{Sc}, $AKIII_{Ec}$, HSK_{Sp}, and *E. faecium* VanS. Molecular weight markers (kDa) are labelled to the left of the gel. B. Detection of ^{32}P in the gel shown in panel A.



Scheme 3.1. Reaction catalyzed by AK and HSK.



Scheme 3.2. Synthesis of aspartate – adenosine bisubstrate compounds **5 - 8**.

Conditions: i) Tetrabutylammonium bromide/KOH; ii) TFA/DCM; iii) $NaHCO_3$; n = 5 – 8.

Chapter 4

Simultaneous *In Vitro* Assay of the First Four Enzymes in the Fungal Aspartate Pathway Identifies a New Class of Aspartate Kinase Inhibitor

David C. Bareich, Ishac Nazi, Gerard D. Wright

4.1. Preface

The experimental work reported in this paper was obtained in collaboration with one other member of the laboratory of Dr. G. Wright. Ishac Nazi was responsible for the cloning of *Saccharomyces cerevisiae* aspartate semialdehyde dehydrogenase and *Schizosaccharomyces pombe* homoserine *O*-acetyl transferase and provided some of each of these proteins used in the screen of the chemical library. The paper has been submitted to Chemistry & Biology, but, at the time of this writing, has not been accepted.

4.2. Summary

The biosynthesis of amino acids derived from aspartate is a target for antifungal agents. We have developed a simultaneous *in vitro* assay of the first four enzymes of the fungal aspartate pathway suitable for high throughput screening of chemical libraries. This pathway includes the generation of unstable or commercially unavailable products/substrates. Our reconstructed pathway assay overcomes this barrier and permits monitoring of enzyme activities initiated with the readily accessible amino acid L-Asp. The pathway assay was empirically optimized such that the first enzyme was rate limiting and the remaining three enzymes were at levels capable of sustaining rates just exceeding that set by the first enzyme. Since the pathway assay monitored production of the last product, inhibitors to any of the four enzymes could be identified. As a proof of concept, we screened a 1,000 compound chemical library against the pathway assay. This screen identified a novel class of 7-chloro-4([1,3,4]thiadiazol-2-ylsulfanyl)-quinoline aspartate kinase inhibitors, that have the potential to act as leads in the development of new antifungal agents. This work has demonstrated the advantages and utility of this pathway assay for inhibitor screening.

4.3. Introduction

The fungal aspartate pathway is required for the biosynthesis of threonine, isoleucine, and methionine (Figure 4.1). The first step in this process is activation of aspartate by phosphorylation catalyzed by aspartate kinase (AK). Aspartate semialdehyde dehydrogenase (ASD) then reduces aspartyl-4-phosphate to aspartate-4-semialdehyde using NADPH. In the next step, homoserine dehydrogenase (HSD) catalyzes the reduction of aspartate semialdehyde to generate the alcohol homoserine using either NADPH or NADH. Homoserine sits at a junction in the pathway: phosphorylation by homoserine kinase commits the carbon skeleton to biosynthesis of threonine and isoleucine, whereas acetylation by homoserine *O*-acetyltransferase (HSAT) commits it to methionine biosynthesis.

The pathway is a good target for new antifungal agents because it is required for fungal viability and is not found in mammals which is why threonine, isoleucine, and methionine must be obtained from the mammalian diet. Additionally, the essentiality of the pathway has been chemically validated as the natural products azoxybacillin (Fujiu et al. 1994), rhizocticin (Kugler et al. 1990), and 2-amino-5-hydroxy-4-oxopentanoic acid (Yamaguchi et al. 1988) have been shown to target homocysteine synthase (Aoki et al. 1994), threonine synthase (Laber et al. 1994), and HSD (Yamaki et al. 1992), respectively, and inhibit the growth of fungi (Figure 4.1). With this in mind, we sought to identify other compounds from a chemical library which could inhibit the fungal

aspartate pathway. This could be achieved by separately screening the library against each enzyme in the pathway. However, this is costly in terms of the chemicals themselves, time, substrates and other materials. Furthermore, 1) it is not possible to screen ASD in the metabolically relevant direction as its substrate, aspartyl-4-phosphate, is unstable, and 2) HSD uses aspartate-4-semialdehyde which is not commercially available and must be chemically synthesized. To obviate these problems we designed a pathway assay whereby the first four enzymes, leading to methionine biosynthesis, are assayed simultaneously (Figure 4.2). Such a pathway assay has the obvious advantages of time and cost savings, but also each additional enzyme screened simultaneously increases the likelihood that an inhibitor may be found from a given set of compounds in a single screen (Wong et al. 1998). A pathway assay may also maintain protein-protein interactions that occur *in vivo* which may be important for the function of the enzymes. Such interactions have been found in the *Escherichia coli* aspartate pathway enzymes aspartate kinase I–homoserine dehydrogenase I and aspartate semialdehyde dehydrogenase which allow substrate channeling of the unstable intermediate aspartyl-4-phosphate (James and Viola 2002). Lastly, the pathway assay also allows the identification of compounds which in principle could be metabolized by the enzymes in the pathway to new forms that have inhibitory activity (Shames et al. 1984).

4.4. Results and Discussion

The first four enzymes of the fungal aspartate pathway were empirically linked together to form the pathway assay in progressive steps starting with *Saccharomyces cerevisiae* AK (AK_{Sc}). AK_{Sc} was assayed for adenosine diphosphate (ADP) production by coupling to pyruvate kinase and lactate dehydrogenase (PK-LDH). Sequential additions of the *S. cerevisiae* enzymes ASD (ASD_{Sc}) and HSD (HSD_{Sc}), which were both assayed by monitoring NADPH oxidation, and *Schizosaccharomyces pombe* HSAT ($HSAT_{Sp}$), assayed for CoASH production, were optimized such that AK_{Sc} was rate limiting and the additional enzymes were present at levels that would sustain rates just exceeding that set by AK_{Sc}. To ensure that these parameters were being met at each optimized step, the steady state kinetic parameters and the L-Thr IC_{50} of AK_{Sc} were assessed prior to optimizing a subsequent step (Table 4.1). With the exception of the ATP K_m values which varied five fold, the remaining parameters for AK_{Sc} were similar after optimizing each step, indicating that AK_{Sc} remained rate limiting. To test the ability of the pathway assay to respond to inhibitors of AK_{Sc}, HSD_{Sc}, and $HSAT_{Sp}$, IC_{50}'s of control inhibitors were determined using the pathway assay (Table 4.1). These include the AK inhibitors L-Thr and β-hydroxynorvaline (Ramos and Calderon 1992), the HSD inhibitor 3-*tert*-butyl-4-hydroxyphenyl sulfide (L. Ejim, G. Wright, unpublished results), and the HSAT inhibitor 2,3-dibromomalonamide (I. Nazi, G. Wright, unpublished results). In all cases, IC_{50}'s were found to be similar to values determined in assays of

the individual enzymes, showing the ability of the pathway assay to identify compounds that inhibit any of the enzymes present.

High throughput screens must be robust to maximize the chances of identifying inhibitory compounds and minimize false positives. One way to visualize the robustness of the assay is to analyze a significant number of positive (100% activity) and negative (0% activity) controls obtained during the screen. The spread of the data values between an arbitrary cutoff of three standard deviations between both positive and negative controls provides a measure of quality of the assay and its usefulness in screening, which we term the screening window (Figure 4.3.A). The statistical parameter Z' reports quantitatively on the data variation and provides a means of assessing the quality of the screen. The Z' factor (see eq (3) in Experimental Procedures) was determined to be 0.65 indicating that the screening window can reliably identify enzyme inhibitors (Zhang et al. 1999).

Choice of compounds to screen is also very important. In general, high throughput screens make use of compounds that are either structurally similar, a focused library, or structurally dissimilar, a small molecule diverse library. The compounds used in this work were obtained from Maybridge plc, which is a chemical supply house which supplies a diverse library. This can be measured by the number of structural clusters with single members and by the number of clusters. The compounds are also drug-like in that the vast majority follow Lipinski's rule of 5. These rules state that drug-like compounds

will have less than 5 hydrogen bond donors, less than 10 hydrogen bond acceptors, a molecular weight less than 500 g/mol, and a calculated Log P less than 5 (Lipinski et al. 2001). Lastly, there is rigorous quality control to ensure that the compounds are pure and stable.

The results of a screen of the pathway assay in duplicate against a 1,000 compound chemical library at a concentration of 10 μM are shown graphically in two ways (Figure 4.3.B and 4.3.C). To assess the reproducibility of the duplicate assay, a plot of the activity remaining in the presence of each compound for each duplicate assay was constructed where the x and y coordinates for each data point are the first and second values, respectively, determined for each compound (Figure 4.3.B). If the duplicates are in perfect agreement they will fall on a line with a slope of one. Three standard deviations below the average value was used as the statistically significant cut off to define a compound as being an inhibitor of the assay. The screen identified two compounds as reproducible inhibitors from the 1,000 compound chemical library.

To assess if the assay showed any particular bias, a frequency distribution histogram of every data point was plotted using 0.5 standard deviation intervals (Figure 4.3.C). Our results show a normal distribution, indicating a predictable and robust assay. The duplicate values for both compounds identified as inhibitors appear at –8 and –9.5 standard deviations, indicating that the duplicates lie within the same 0.5 standard

deviation interval and their levels of inhibition are significant and potent at the 10 μM concentration used in the screen.

The structure of one of the compounds identified in the screen, *N*-[5-(7-chloro-quinolin-4-ylsulfanyl)-[1,3,4]thiadiazol-2-yl]-2-phenoxy-nicotinamide (**1**), is shown in Table 4.2. The second hit showed unusual behavior in further analyses and is not discussed here. IC_{50}'s were determined for all sequential combinations of the pathway beginning with AK_{Sc}, as well as the assay for ADP production by AK_{Sc}, to determine which enzyme was inhibited by **1** (Table 4.3). Since IC_{50} values were obtained from all sequential combinations of the pathway and the values were within 4 fold of each other, the target of inhibition by **1** was concluded to be AK_{Sc}.

The mechanism of AK_{Sc} inhibition and K_i values of **1** were determined by assaying for NADPH oxidation using an AK_{Sc}-ASD_{Sc} coupled assay. Double reciprocal plots for the inhibition of AK_{Sc} by **1** versus both substrates revealed that **1** is a non-competitive inhibitor of ATP (K_{ii} 10 ± 1.4 μM, K_{is} 12 ± 3.1 μM) and an uncompetitive inhibitor of L-Asp (K_{ii} 5.1 ± 0.31 μM) (Figure 4.4). Compound **1** had no effect on the growth of *S. cerevisiae* DL1, *Candida parapsilosis* ATCC 90018, and *Candida albicans* ATCC 90028 in minimal RPMI liquid media at concentrations up to 64 μg/mL.

Structural analogs of **1** were identified in an *in silico* substructure search of a 50,000 compound chemical library from Maybridge plc. IC_{50}'s were determined using the AK_{Sc}-PK-LDH assay for each analog to determine what structural features were important for inhibition of AK_{Sc} (Table 4.2). Two analogs of **1** that maintain the core 7-chloro-4([1,3,4]thiadiazol-2-ylsulfanyl)-quinoline structure, **2** and **3**, showed IC_{50}'s similar to the parent compound indicating that the 2-phenoxypyridine group can be replaced by other heterocycles. This portion of the molecule is important for enzyme affinity as **4**, in which the 2-phenoxypyridine is replaced by an isobutene group, shows no interaction with the enzyme at 30 μM, the highest concentration tested (Table 4.2). Similarly, the 4-thio-7-chloroquinoline group was also essential as the replacement with a cyclopropane group (compound **5**) results in loss of activity (Table 4.2).

4.5. Significance

We designed and optimized a pathway assay of the first four enzymes in the fungal aspartate pathway for use in high throughput screening of chemical libraries. The aspartate pathway is a good target for new antifungal agents as it is not found in mammals and is required for fungal viability shown by several natural products which inhibit the pathway. This work has demonstrated that this pathway assay is amenable to high throughput screening, which has several advantages over separately screening each enzyme including time and cost improvements, but also the ability to screen ASD_{Sc} and HSD_{Sc} in the metabolically relevant direction. This pathway assay enabled the

identification of a new class of inhibitors of fungal AK and structure activity analysis revealed structural constraints for inhibition. These are the first reported non-amino acid inhibitors of fungal AK and as such could serve as leads in new antifungal compound development.

4.6. Experimental Procedures

4.6.1. Cloning, Expression, and Purification of Fungal Aspartate Pathway Enzymes.

Cloning, expression, and purification of AK_{Sc} and HSD_{Sc} are described elsewhere (Jacques et al. 2001; Bareich et al. 2003).

The *HOM2* gene encoding ASD_{Sc} was amplified from *S. cerevisiae* genomic DNA using the polymerase chain reaction and the oligonucleotide primers 5'-GGTGGTCATATGGCTGGAAAGAAAATTGCTGG and 5'-CCGCTCGAGGGATCCTTAAATCAAGTTTCTTGCTAGTAAGATTTCGG. The amplified fragment was cloned into the *Nde* I and *Xho* I restriction enzyme sites of pET28 using standard techniques. The resulting DNA sequence confirmed plasmid, pET28 + ASD_{Sc}, was transformed into *E. coli* BL21(DE3) cells allowing expression of ASD_{Sc} with an N-terminal hexa-histidine tag.

The His-tagged enzyme was expressed in *E. coli* BL21(DE3)/pET28 + ASD_{Sc} by growing two 1 L cultures in LB supplemented with 50 μg/mL kanamycin to an OD_{600} of 0.6 at 37°C. The cultures were cooled in an ice-water bath to 16 °C, isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM, and incubated overnight at 16 °C in an orbital shaker at 250 rpm. Cells were harvested by centrifugation at 13,000 x g for 10 min, the cell pellet was resuspended in 20 mL of 20 mM 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES) pH 8.0, and lysed with 3 passes through a French Pressure cell at 10,000 psi in the presence of 1 mM phenylmethylsulfonyl fluoride. Following centrifugation for 10 min at 43,000 x g, the supernatant was applied on to a 5 mL Ni NTA Agarose column, the column was washed with 20 mM HEPES pH 8.0 until the OD_{280} decreased to less than 100 mAu, and the bound protein was eluted with a gradient of 0 to 100 mM imidazole in 20 mM HEPES pH 8.0. Active fractions were identified immediately, pooled and dialyzed over night against 4 L of 20 mM HEPES pH 8.0. The dialyzed enzyme was concentrated and frozen at –80°C with 10% glycerol.

The *MET2* gene encoding $HSAT_{Sp}$ was amplified from *S. pombe* genomic DNA using the oligonucleotide primers 5'-GGGAATTCCATATGGAATCTCAATCTCCGATTGAATCAATTGTCTTTAC and 5'-CGCGGATCCAAGCTTTTACCAGGAGGTTATGTCTTCCATTTCTC. The amplified fragment was cloned into the *Nde* I and *Hind* III restriction enzyme sites of

pET28 using standard techniques and the DNA sequence was verified. The resulting plasmid, pET28 + $HSAT_{Sp}$, was transformed into *E. coli* BL21(DE3) cells allowing the expression of $HSAT_{Sp}$ with an N-terminal hexa-histidine tag.

The His-tagged enzyme was expressed in *E. coli* BL21(DE3)/pET28 + $HSAT_{Sp}$ in 1 L of LB supplemented with 50 μg/mL kanamycin to an OD_{600} of 0.6 at 37°C. The cultures were cooled in an ice-water bath to 16 °C, IPTG was added to a final concentration of 1 mM, and were incubated for 2 h at 16°C in an orbital shaker. The cultures were harvested by centrifugation at 13,000 x g for 10 min, and the cells from four 1L cultures resuspended in 30 mL of 20 mM HEPES pH 8.0, and lysed with 3 passes through a French Pressure cell at 10,000 psi. Cell debris was pelleted at 43,000 x g for 10 min and the supernatant was loaded on a 15 mL Ni NTA Agarose column. The column was washed until the OD_{280} decreased to less than 100 mAu, and the bound $HSAT_{Sp}$ was eluted with 10 mL of 100 mM imidazole in 20 mM HEPES pH 8.0 directly onto a 20 mL Q Sepharose column, which was washed similarly and eluted with a gradient of 0 to 400 mM NaCl in 20 mM HEPES pH 8.0. $HSAT_{Sp}$ active fractions were pooled and found to yield 20 mg of pure $HSAT_{Sp}$.

4.6.2. ADP Production Assay

AK_{Sc} activity was determined by coupling production of ADP with the lactate dehydrogenase dependent oxidation of reduced NADH in the presence of phosphoenol

pyruvate and pyruvate kinase. Reaction mixtures contained 7 mM ATP, 0.33 U pyruvate kinase, 0.66 U lactate dehydrogenase, 2.5 mM phosphoenol pyruvate, 1 mM NADH, 10 mM KCl, 40 mM $MgSO_4$, 100 mM HEPES pH 7.5 in a final volume of 100 µL contained in a 96 well flat bottom microtitre plate. A 6 minute pre-incubation was used to ensure that any contaminating ADP was regenerated to ATP as well as allowing for temperature equilibration to 30 °C. The reactions were initiated with the addition of at least 25 mM L-aspartate and monitored at 340 nm in a Molecular Devices Spectramax microtitre platereader. For steady state kinetics, eight substrate concentrations in duplicate were used, ranging from 0.4 to 7 mM for ATP and 0.5 to 30 mM L-Asp. Data were fit to eq (1) for Michaelis-Menten kinetics using the computer program GraFit (Leatherbarrow 2001).

$$\upsilon = \frac{V_{\max} S}{(K_m + S)} \qquad (1)$$

4.6.3. L-Threonine IC_{50} Determinations

Assays were similar to assays for ADP production, with the addition of L-threonine concentrations ranging from 0.05 to 150 mM, ATP and L-Asp were present at 5 and 12.5 mM, respectively. Data were fit to the four parameter eq (2), using GraFit (Leatherbarrow 2001) to solve for the IC_{50}, where A = minimum response plateau, D = maximum response plateau, I = concentration of inhibitor, and S = slope factor.

$$y = \frac{A - D}{1 + \left(\frac{I}{IC_{50}}\right)^{S}} + D \qquad (2)$$

4.6.4. NADPH Oxidation Assay

NADPH oxidation by ASD_{Sc} separately or with HSD_{Sc} was monitored at 340 nm ($\varepsilon_{340\,nm}$ = 6300 $M^{-1}cm^{-1}$). Conditions for this assay are identical to those for ADP production with the substitution of ASD_{Sc} alone or in combination with HSD_{Sc} for PK-LDH.

4.6.5. Assay for CoASH Production

$HSAT_{Sp}$ production of CoASH was by monitored by *in situ* titration of 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) (Ellman 1959). Production of the mixed disulfide of CoASH-5-thio-2-nitrobenzoic acid and release of the chromophoric thiolate was monitored at 412 nm ($\varepsilon_{412\,nm}$ = 13600 $M^{-1}cm^{-1}$), Figure 4.2. The assay was buffered with 100 mM HEPES pH 7.0, and contained 40 mM $MgSO_4$, 10 mM KCl, 0.8 mM ATP, 1.2 mM NADPH, and 0.2 mM AcCoA and the optimized amounts of AK_{Sc}, ASD_{Sc}, HSD_{Sc}, and $HSAT_{Sp}$. The pathway assay reactions were initiated by the addition of a mixture of L-Asp and DTNB, such that final concentrations were 6 and 0.22 mM,

respectively. Assays were monitored at 412 nm for a minimum of 5 min at 30°C. Similar assays were used for the purification of $HSAT_{Sp}$ except reactions were initiated with L-homoserine and DTNB, avoiding the need for the other pathway enzymes and their substrates.

4.6.6. Optimization of the Pathway Assay

The amount of AK_{Sc} yielding an easily measured progress curve (slope approximately 20 – 30 times above background) that was linear for at least 5 minutes was determined by titrating AK_{Sc} and assaying for ADP production. The amount of ASD_{Sc} sufficient to just exceed the rate established by AK_{Sc} was determined empirically by varying ASD_{Sc} while keeping AK_{Sc} constant and assaying for NADPH oxidation. The amount of HSD_{Sc} was optimized in a similar manner as for ASD_{Sc}, however, the observed slope was twice that found for ASD_{Sc} as the stoichiometry of NADPH consumption had doubled due to the added dehydrogenase activity of HSD_{Sc}. To reduce expense, the concentration of NADPH was kept to a minimum, 1.2 mM, without becoming rate limiting. The amount of $HSAT_{Sp}$ was optimized in a similar manner using the assay for CoASH production. Observed activity was found to decrease with increasing concentrations of DTNB, likely due to inactivation of one of the pathway enzymes by DTNB, therefore this reporter molecule was added at a concentration just exceeding the limiting substrate concentration with the initiating substrate L-Asp. After each additional

enzyme was optimally linked, the AK_{Sc} steady state kinetic parameters along with the L-Thr IC_{50} were determined to confirm that the rate limiting step remained AK_{Sc}.

4.6.7. Enzyme Screening

Compounds from the Maybridge collection were dissolved at 200 μM in dimethyl sulfoxide in 96 well microtitre plates. Compounds, sufficient for a 10 μM final concentration, and master mix containing buffer, Mg^{2+}, enzymes and substrates were transferred to the assay plate and pre-incubated 15 min at 30 °C in concentrations identical to the assay conditions for CoASH production. Reactions were initiated by the addition of a mixture of L-Asp and DTNB, such that final concentrations were 6 and 0.22 mM, respectively. Assays were monitored at 412 nm for 5 min at 30 °C.

4.6.8. Data Analysis and Characterization of Inhibitory Compounds

The quantitative measure of the quality of the assay, called the Z' factor, was calculated for the assay, eq 3, (Zhang et al. 1999). IC_{50} values for the various inhibitory compounds were determined using equation 2.

$$Z'\ \ factor = 1 - \left[\frac{\left(3\sigma_{high} + 3\sigma_{low}\right)}{\left(average_{high} - average_{low}\right)} \right] \qquad (3)$$

The mechanism of inhibition of AK_{Sc} by hits derived from the primary screen was determined along with their K_i values. To assess the mechanism of inhibition, double reciprocal plots were generated with individual lines fit to a simple double reciprocal model, eq 4 and the type of inhibition confirmed by F-test comparing global fits of the data to models of competitive, eq 5, uncompetitive, eq 6, and noncompetitive inhibition, eq 7. Inhibition constants were obtained from the global fit to the appropriate model.

$$\frac{1}{v} = \frac{1}{V_{\max}} + \frac{K_m}{V_{\max} S} \tag{4}$$

$$\upsilon = \frac{V \max S}{\left(K_m \left(1 + \frac{I}{K_{is}} \right) + S \right)} \tag{5}$$

$$\upsilon = \frac{V_{\max} S}{\left(K_m + S \left(1 + \frac{I}{K_{ii}} \right) \right)} \tag{6}$$

$$\upsilon = \frac{V_{\max} S}{\left(K_m \left(1 + \frac{I}{K_{is}} \right) + S \left(1 + \frac{I}{K_{ii}} \right) \right)} \tag{7}$$

4.7. Acknowledgements

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Table 4.1. Optimization and inhibition of enzyme throughput. Inhibitor controls and AK_{Sc} steady state kinetic parameters for each step in the development of the pathway assay.

	ATP		L-Asp				
Linked Enzymes	K_m (mM)	k_{cat} (s^{-1})	K_m (mM)	k_{cat} (s^{-1})	Compound	IC_{50} (mM)	Enzyme Inhibited
AK_{Sc}-PK-LDH	1.3	40	3.3	36	L-Thr	3.4	AK_{Sc}
AK_{Sc}-ASD_{Sc}	0.50	49	2.5	48	L-Thr	4.5	AK_{Sc}
AK_{Sc}-ASD_{Sc}-HSD_{Sc}	2.3	50	3.4	43	L-Thr	3.6	AK_{Sc}
AK_{Sc}-ASD_{Sc}-HSD_{Sc}-$HSAT_{Sp}$	0.53	45	3.4	50	L-Thr	4.0	AK_{Sc}
AK_{Sc}-ASD_{Sc}-HSD_{Sc}-$HSAT_{Sp}$					β-hydroxy-norvaline	5.3	AK_{Sc}
AK_{Sc}-ASD_{Sc}-HSD_{Sc}-$HSAT_{Sp}$					B666	7.3	HSD_{Sc}
AK_{Sc}-ASD_{Sc}-HSD_{Sc}-$HSAT_{Sp}$					L311	4.8	$HSAT_{Sp}$

Table 4.2. SAR of 7-chloro-4([1,3,4]-thiadiazol-2-ylsulanyl)-quinoline aspartate kinase inhibitors identified in the screen. Where no inhibition is indicated the maximum concentration tested was 30 μM.

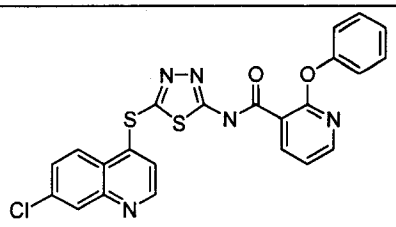
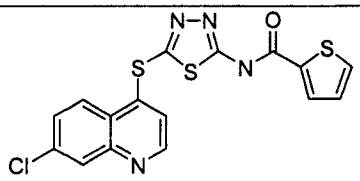
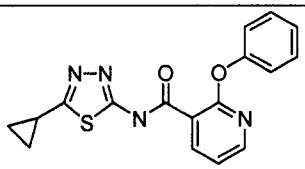
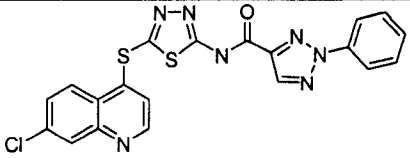
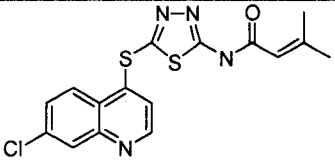
 **1** 3.1 ± 0.78 μM	
 **2** 3.6 ± 0.78 μM	 **5** No Inhibition
 **3** 1.6 ± 0.66 μM	
 **4** No Inhibition	

Table 4.3. Identification of the enzyme inhibited by **1**.

Linked Enzymes	IC_{50} (μM)
AK_{Sc}-ASD_{Sc}-HSD_{Sc}-$HSAT_{Sp}$	3.1 ± 0.52
AK_{Sc}-ASD_{Sc}-HSD_{Sc}	12 ± 4.3
AK_{Sc}-ASD_{Sc}	12 ± 2.5
AK_{Sc}-PK-LDH	3.1 ± 0.78

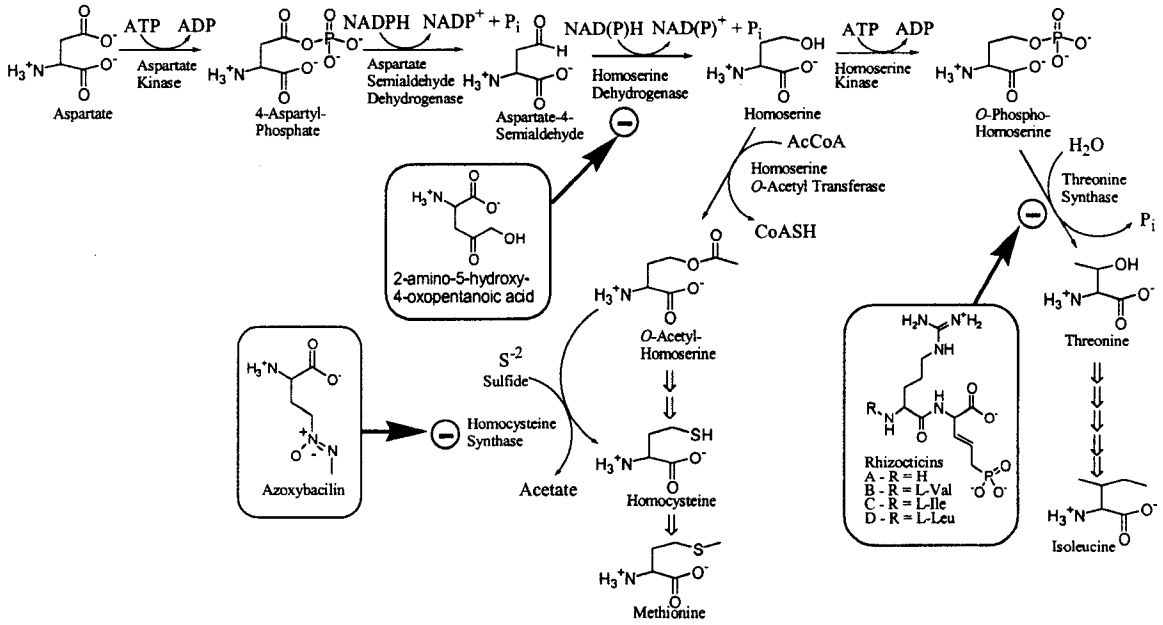


Figure 4.1. The fungal aspartate pathway showing the natural product inhibitors azoxybacillin, the rhizocticins, and 2-amino-5-hydroxy-4-oxopentanoic acid.

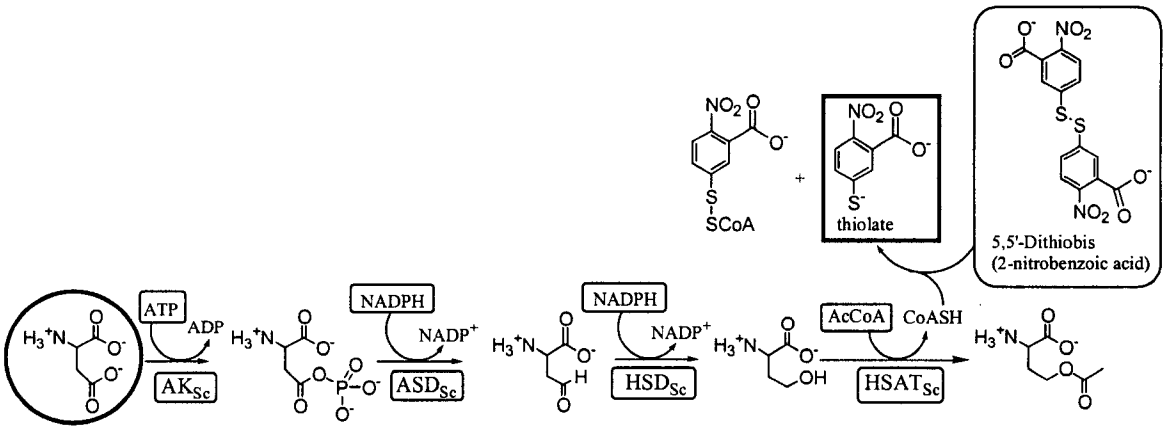
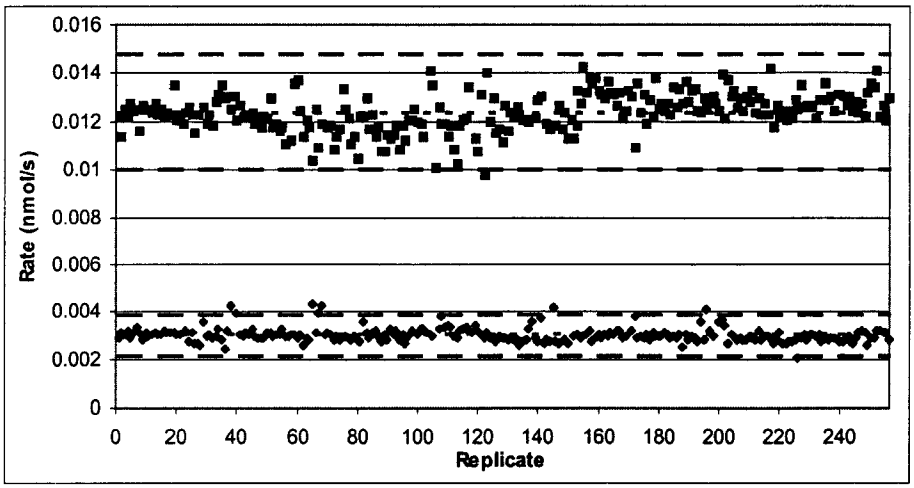


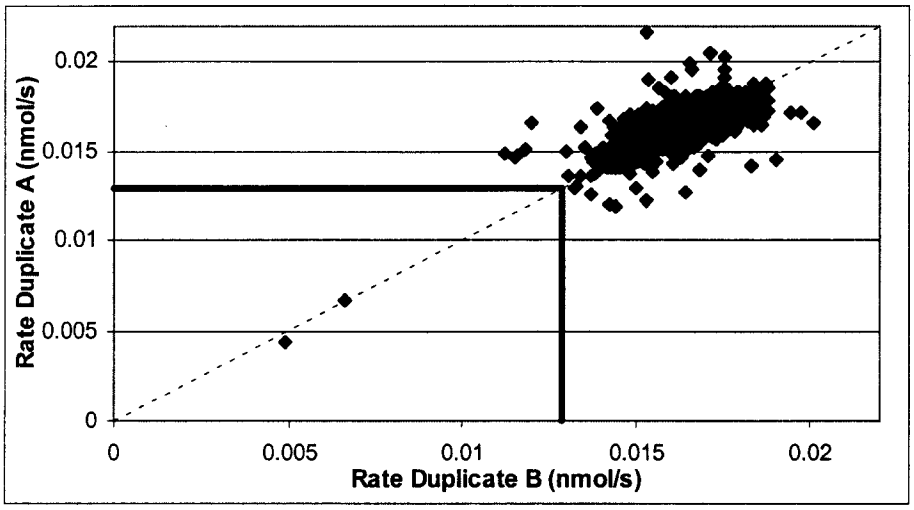
Figure 4.2. The assay of the first four enzymes in the fungal aspartate pathway.

Figure 4.3. Results of the enzyme screening. A. Plot of 100 (■) and 0% (♦) activity controls for the high throughput screen. Dashed lines indicate the average for the controls, heavy dashed lines indicate three standard deviations. The screening window is located between the inner 3 standard deviation lines for the 100 and 0% activity controls, at approximately 0.01 and 0.004 nmol/s, respectively. B. Results of the screen of 1,000 compounds against the pathway assay. Duplicate values for each compound are plotted on opposing axes, if the duplicates are in agreement they should fall on a line with a slope of 1 (dashed line). Three standard deviations below the average result is considered to be statistically significant inhibition and is demarked by thick lines. C. Frequency distribution of high throughput screening results. The results are plotted in 0.5 sigma intervals and resemble a normal curve (thin line). The two hits can be seen at –8 and –9.5 sigma and are indicated by the arrows.

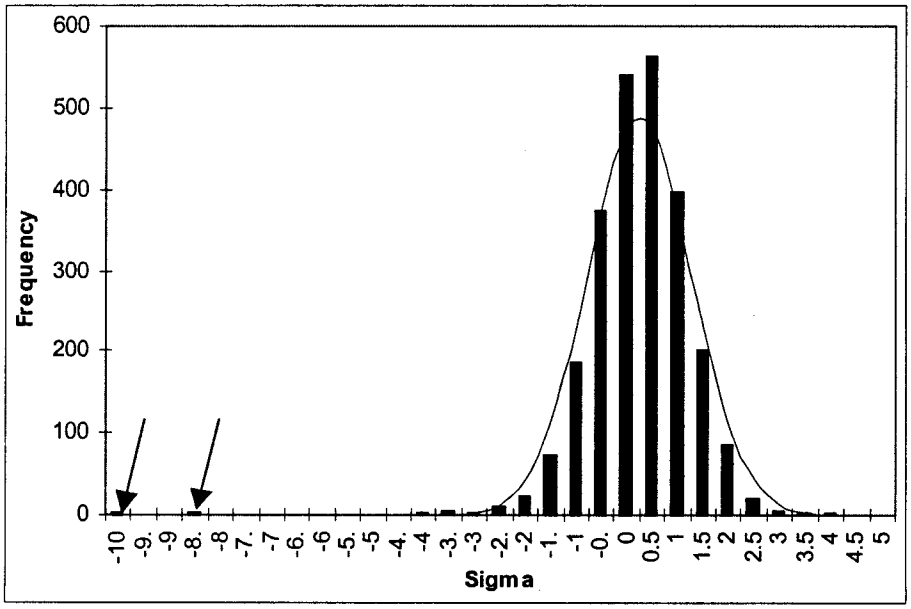
A


Rate (nmol/s)
0.016
0.014
0.012
0.01
0.008
0.006
0.004
0.002
0
0 20 40 60 80 100 120 140 160 180 200 220 240
Replicate

B


Rate Duplicate A (nmol/s)
0.02
0.015
0.01
0.005
0
0 0.005 0.01 0.015 0.02
Rate Duplicate B (nmol/s)

C


Frequency
600
500
400
300
200
100
0
-10 -9. -9 -8. -8 -7. -7 -6. -6 -5. -5 -4. -4 -3. -3 -2. -2 -1. -1 -0. 0 0.5 1 1.5 2 2.5 3 3.5 4 4.5 5
Sigma

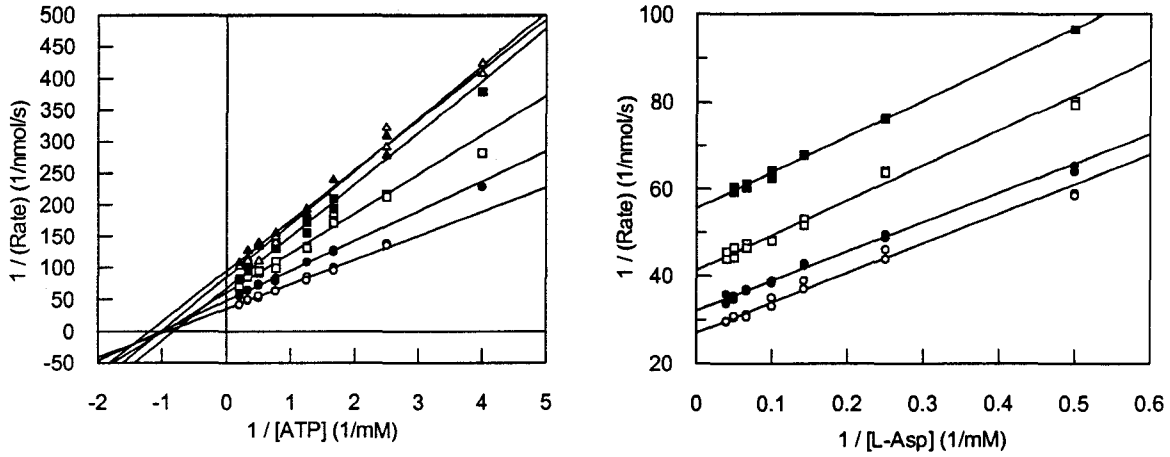


Figure 4.4. Double reciprocal plots for the inhibition of AK_{Sc} by **1** versus L-Asp and ATP. The activity of AK_{Sc} was monitored by coupling aspartyl-4-phosphate production to ASD_{Sc} as described in Experimental Procedures.

Chapter 5

Discussion and Future Work

5.1 Contributions Towards Understanding How Aspartate Kinase Catalyzes Phosphoryl Transfer

The aim of work presented in this thesis was to deepen the understanding of how AK functions and to develop inhibitors to AK and the enzymes in the aspartate pathway in general. Chapter 2 discussed the assessment of amino acid exchanges in AK_{Sc}, as well as the effect of pH on the kinetic parameters of several of the variants. These studies were the first demonstration of amino acids in AK_{Sc} important for catalysis, whereas many studies have looked at amino acids important for the allosteric inhibition of AK_{Sc}. While the studies in Chapter 2 are a good beginning to understanding how aspartate kinase catalyzes phosphoryl transfer, they by no means provide a full understanding. An x-ray crystal structure of an aspartate kinase would greatly aid in this understanding as it would provide information about the amino acids in the active site which could be used to direct further studies. A crystal structure would also be useful in understanding how L-Thr regulates AK_{Sc}. Such knowledge is needed not only for the rational design of inhibitors to AKs, but also for the genetic engineering of AK to improve the nutritional value of plants by increasing the content of L-Thr, L-Ile, and L-Met.

Chapter 3 discussed the evidence for a direct path of phosphoryl transfer from ATP to aspartate and the lack of ATPase activity in the absence of aspartate. Although this lack of ATPase activity suggests that a metaphosphate intermediate is not present, it does not rule it out, as the presence of aspartate may be required for metaphosphate

formation. Thus, conclusive evidence of the mechanism of phosphoryl transfer was not obtained and would required the use of positional isotope effect (PIX) studies. We had every intention of doing PIX studies, but were prevented by the onerous chemical synthesis of ATP with ^{18}O specifically labelling the four γ phosphate oxygens. Although Dr. Kalinka Koteva was able to recover ATP from the synthesis, the percentage labelling was insufficient for our purposes. Revisiting this chemistry to make the labelled ATP would allow PIX experiments which would yield information about the mechanism of phosphoryl transfer.

5.2 Inhibition of Aspartate Kinase

Two strategies were used in the pursuit of inhibitors to AK and the other enzymes in the aspartate pathway. A rational design which is based on some prior knowledge of the enzyme and the reaction it catalyzes, and a screening strategy in which an assay is used to screen a series of compounds for their ability to inhibit the enzyme. Both strategies require some luck. In a rational design strategy, luck is required to translate the prior knowledge about the enzyme effectively into a compound capable of inhibiting the enzyme. A screening strategy lives and dies on the assay and the compounds in the library, with luck the library contains a compound which inhibits the enzyme and the assay is capable of detecting the inhibition at the compound concentration used in the screen.

5.2.1 Rational Design of Inhibitors to Aspartate Kinase

Rational design of enzyme inhibitors usually requires knowledge of the enzyme active site and how substrates and/or transition states are bound. Such knowledge is sorely lacking for AKs. However, another method to rationally design inhibitors to enzymes with multiple substrates/products is being increasingly used. Inhibitors are designed to mimic multiple substrates/products and their spatial orientation by covalently linking substrate/products or analogs of them. When knowledge about the separation distance between the substrates/products is not known, variable linker lengths have been used. Chapter 2 discusses the bisubstrate compounds designed to inhibit AKs which were found to inhibit $AKIII_{Ec}$ but not AK_{Sc}. The functional discrimination of the bisubstrate compounds by the two AKs was surprising considering they both randomly bind identical substrates and directly transfer phosphate to aspartate. While this discrimination was surprising, it could be useful to create compounds which specifically inhibit bacterial or fungal AKs. Such compounds could in turn lead to drugs with antibacterial or antifungal activities.

Investigating how the bisubstrate compounds can inhibit the bacterial AK and not the fungal AK could reveal ways to create compounds that inhibit both kinases. For example, the fungal AK may require greater functionality in the linker region, which evidently is not required in bacterial AKs. As well, it would be interesting to determine if

greater inhibition could be achieved with longer linker lengths for $AKIII_{Ec}$ or shorter lengths for AK_{Sc}.

The bisubstrate compounds provided no inhibition of HSK_{Sp} despite significant similarities between AKs and HSKs. It was proposed that the sp^2 hybridization of the C4 carbon of homoserine is selected for by the active site of HSKs. Testing this with bisubstrate compounds employing sp^2 geometry at that position could yield compounds which inhibit HSKs. If AKs do not select against sp^2 geometry at the C4 position of the amino acid portion, such bisubstrate compounds could inhibit both AKs and HSKs and provide a lead for antibiotics less likely to be resisted by target alteration due to multiple targets being inhibited.

5.2.2 Screening a Chemical Library for Inhibitors to the First Four Enzymes of the Aspartate Pathway

The second strategy to obtain enzyme inhibitors as indicated above, is to screen chemical libraries using an activity assay for the enzyme. This can be an excellent way to identify inhibitors with greater chemical diversity and is a favourable strategy when little is known about the enzyme target. To maximize the chances of identifying inhibitors a large number of compounds should be screened.

When one considers the cost and effort needed to obtain chemical libraries it is wise to maximize their utility. To do this, assays need to be designed that are robust, statistically significant, repeatable, and optimized to minimize time and cost. One approach to identify the greatest number of inhibitors from a chemical library in a single assay is to increase the number of targets. This could be accomplished using whole cell assays or using a single assay to monitor several enzymes, an excellent example is the assaying metabolic pathways.

Chapter 4 discussed the optimization of an assay of the first four enzymes in the aspartate pathway leading to the biosynthesis of L-Met. As a proof of principle a 1,000 compound library was screened and found to contain two compounds which inhibited the assay with statistical significance. Both compounds were found to inhibit AK_{Sc}, the first enzyme in the pathway assay. Follow-up studies with compounds of similar structure revealed that one of the compound families had some interesting structure – activity relationships, while members of the other family did not inhibit the assay. These studies provided the first non-amino acid inhibitors of any AK.

An extension of this work would be the assaying of much larger libraries so that inhibitors to the other enzymes in the pathway assay could be identified. As well, the inhibitors identified in the screen of the 1,000 compound library should be assessed for their ability to inhibit $AKIII_{Ec}$ and HSK_{Sp} to estimate their spectrum of activity. With this in mind, efforts to clone and express aspartate pathway enzymes from other bacteria and

fungi, from both pathogens and non-pathogens, as well as plants, would greatly aid in characterizing enzyme inhibitors as potential antibiotic, antifungal or herbicidal lead compounds.