Available online at www.sciencedirect.com
SCIENCE DIRECT®
Bioorganic & Medicinal Chemistry
ELSEVIER
Bioorganic & Medicinal Chemistry 12 (2004) 807–815

# Small molecule functional discrimination of the kinases required for the microbial synthesis of threonine and isoleucine

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

*Antimicrobial Research Centre, Department of Biochemistry, McMaster University, 1200 Main St. W. Hamilton, ON, Canada L8N 3Z5*

Received 14 May 2003; accepted 23 October 2003

**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.


## 1. Introduction

Fungi and bacteria can biosynthesize L-threonine and L-isoleucine using a metabolic pathway that 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.[1] These kinases discriminate amino acid substrates that differ only in the oxidation state of a single carbon, albeit with significantly different chemical properties, Scheme 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.[1] AKs catalyze the transfer of the γ-phosphate of adenosine triphosphate, (ATP) to L-Asp producing adenosine diphosphate (ADP) and 4-phosphoaspartate, Scheme 1. The 4-phosphoaspartate product is unstable and has been shown to have a half life of approximately 1 h at pH 8.0.[2] 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 that 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.[3,4] AKI-$HSDI_{Ec}$ and $AKIII_{Ec}$ have been shown to catalyze phosphotransfer to a structurally diverse set of L-Asp analogues.[2,5] 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.[6,7]

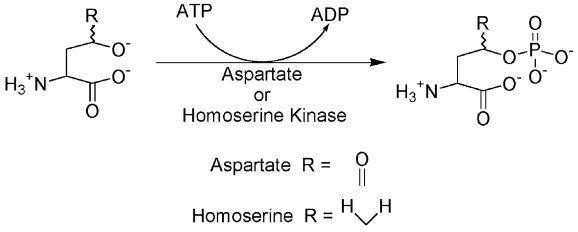
HSKs catalyze the first committed step towards the biosynthesis of threonine and isoleucine. The reaction they catalyze is similar to that catalyzed by AKs with the difference that the phosphoryl acceptor on L-homoserine is an alcohol rather than a carboxylic acid, Scheme 1. This distinction results 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

*Keywords:* Amino acid kinase; Phosphoryl transfer mechanism; Inhibitor; Bisubstrate.
*Abbreviations:* AK, aspartate kinase; HSK, homoserine kinase; $AK_{Sc}$, *Saccharomyces cerevisiae* AK; ATP, adenosine triphosphate; ADP, adenosine diphosphate; AKI-$HSDI_{Ec}$, *Escherichia coli* AKI—homoserine dehydrogenase I; AKII-$HSDII_{Ec}$, *E. coli* AKII-HSDII; $AKIII_{Ec}$, *E. coli* AKIII; $HSK_{Ec}$, *E. coli* HSK; HEPES, 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid; PMSF, phenylmethylsulfonyl fluoride; SDS, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid; $HSK_{Mj}$, *Methanococcus jannaschii* HSK; $HSK_{Sp}$, *Schizosaccharomyces pombe* HSK; $ASD_{Sc}$, *S. cerevisiae* aspartate semialdehyde dehydrogenase.
* Corresponding author. Tel.: +1-905-525-9140x22454; fax: +1-905-525-9033; e-mail: wrightge@mcmaster.ca

0968-0896/$ - see front matter © 2003 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmc.2003.10.042

to AKI-HSDI$_{Ec}$ and AKIII$_{Ec}$.[8] Substrates bind to HSK$_{Ec}$ in a preferred order random mechanism where ATP is preferentially bound before L-homoserine.[9] Crystal structures of *Methanococcus jannaschii* homoserine kinase, (HSK$_{Mj}$), with ADP,[10] as well as with homoserine[11] 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.




**Scheme 1.** Reaction catalyzed by AK and HSK.

## 2. Results and discussion

### 2.1. 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 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*.[12,13] 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,[5] 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.

**Table 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 |

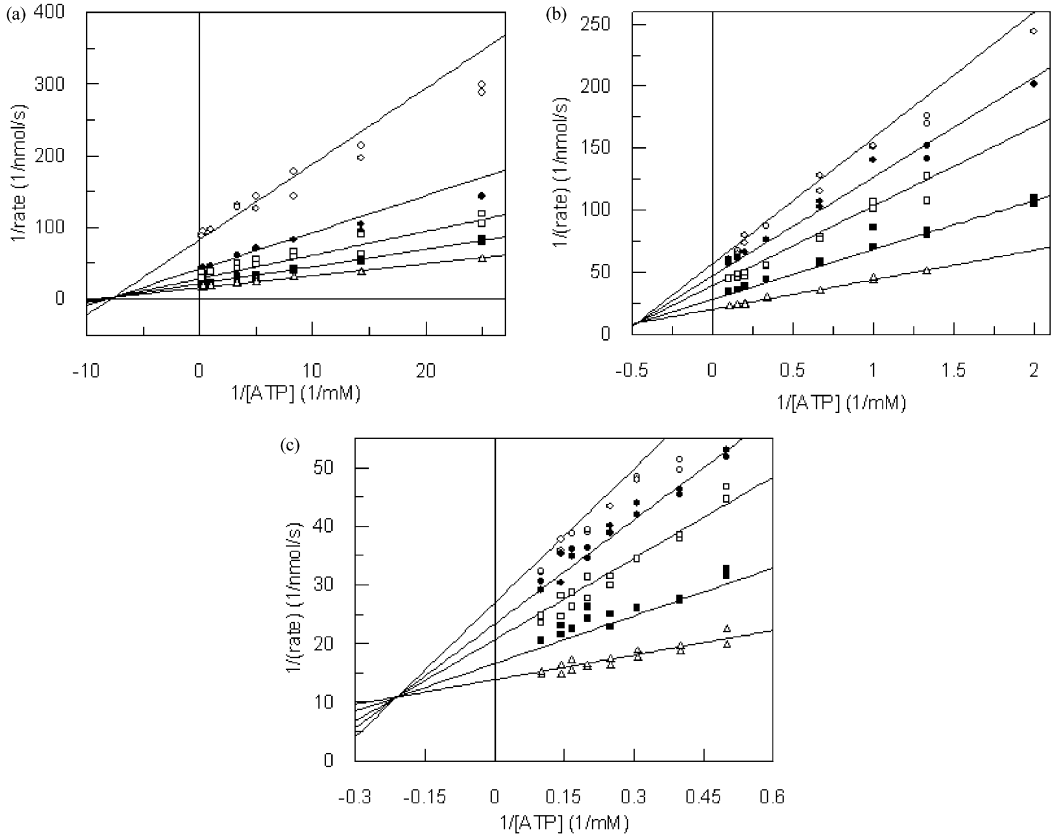



**Figure 1.** Kinases of the aspartate pathway follow a common ternary complex mechanism. Double reciprocal plots of A. HSK$_{Sp}$ using homoserine concentrations of 0.1 (○), 0.25 (●), 0.5 (□), 1 (■), and 5 mM (△), B. AKIII$_{Ec}$ using L-Asp concentrations of 2.25 (○), 3 (●), 4 (□), 8 (■), and 25 mM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

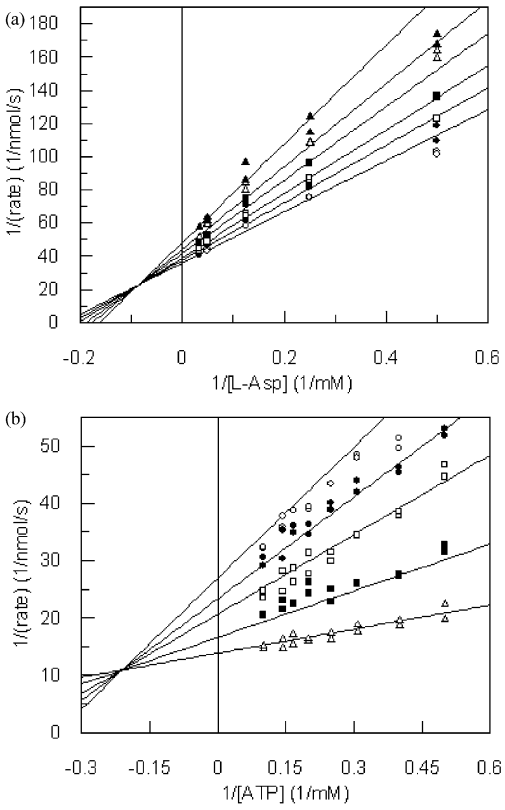



**Figure 2.** Product inhibition analysis of $AK_{Sc}$ with respect to L-Asp, A., and ATP, B., using the AK-ASD assay. ADP concentrations are 0 (○), 0.2 (●), 0.4 (□), 0.7 (■), 1 (△), and 1.5 mM (▲).

### 2.2. 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 1, showing that the enzymes use a ternary complex mechanism and not a ping pong mechanism consistent with literature precedent for $AKIII_{Ec}$[6] and *E. coli* HSK.[9] 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 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 2, suggests that L-Asp and ATP are bound in a random order, which agrees with the order of binding established for $AKIII_{Ec}$[6] and the AK domain of AKI-$HSDI_{Ec}$.[7] 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.[9] 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 labeled with $^{32}$P (Fig. 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,[14] was labeled 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 $AK_{Sc}$,

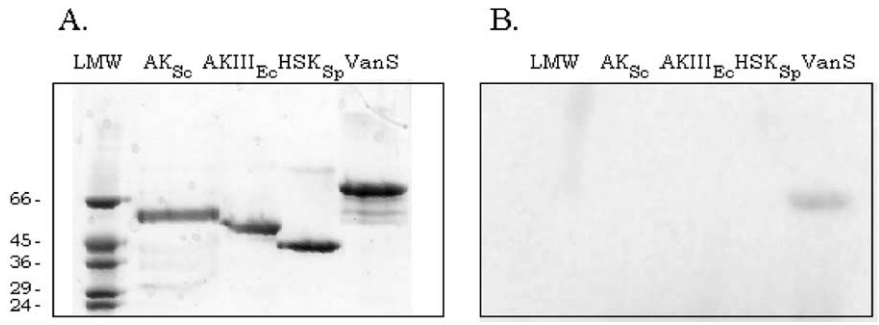
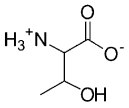
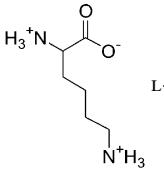
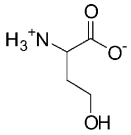
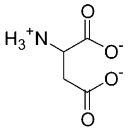
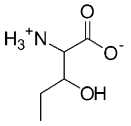
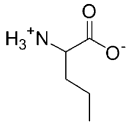



**Figure 3.** Aspartate pathway kinases are not phosphorylated by ATP. A. Coomassie stained gel of $AK_{Sc}$, $AKIII_{Ec}$, $HSK_{Sp}$, and *E. faecium* VanS. Molecular weight markers (kDa) are labeled to the left of the gel. B. Detection of $^{32}$P in the gel shown in panel A.

**Table 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 |

$AKIII_{Ec}$, and $HSK_{Sp}$ are similar in all respects. Control incubations of the labeled ATP with kinase inactivated by boiling established the intensity, location, and size of the ATP spot. Incubations of the kinases with either $\gamma$-$^{32}$P-ATP or $\alpha$-$^{32}$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 $\alpha$-$^{32}$P-ATP incubations as an increase in the ADP spot, whereas the $\gamma$-$^{32}$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 labeling 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.

### 2.3. Small molecule inhibitors of $AK_{Sc}$, $AKIII_{Ec}$, and $HSK_{Sp}$

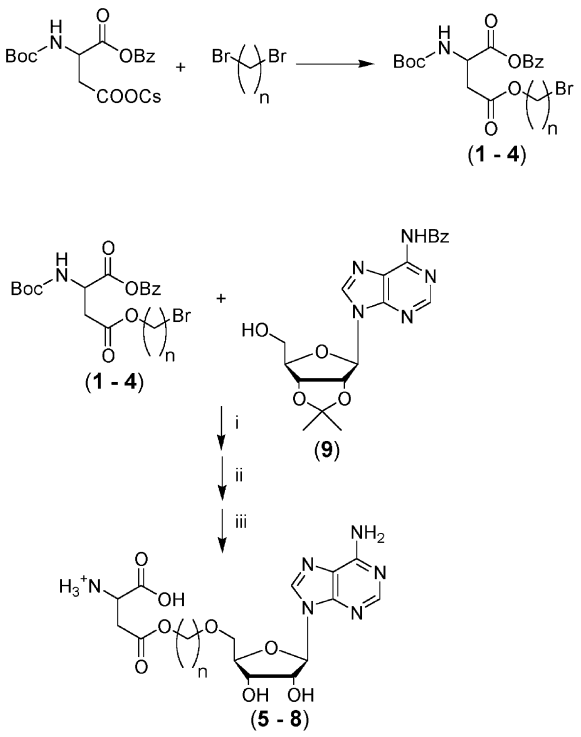
A summary of the $AK_{Sc}$, $AKIII_{Ec}$, and $HSK_{Sp}$ $IC_{50}$ values for the in vivo allosteric inhibitors L-Thr ($AK_{Sc}$) and L-Lys ($AKIII_{Ec}$), aspartate pathway substrates L-homoserine and L-Asp, and amino acid analogues hydroxynorvaline and L-norvaline are shown in Table 2. The $IC_{50}$ values for the in vivo allosteric inhibitors were within 3 fold of previously published values.[4,15] The $AK_{Sc}$ $IC_{50}$ value for hydroxynorvaline compares favorably to the previously reported values at fixed concentrations of hydroxynorvaline.[4,16] The lack of inhibition of $AKIII_{Ec}$ by hydroxynorvaline may reflect differential binding of small molecules by the two AKs.

### 2.4. 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 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 2. One or more of

**Table 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 |




**Scheme 2.** Synthesis of aspartate– adenosine bisubstrate compounds **5**–**8**. Conditions: (i) Tetrabutylammonium bromide/KOH; (ii) TFA/DCM; (iii) $NaHCO_3$; n = 5–8.

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, 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 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

**Table 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 5.** PCR primers used in cloning $AK_{Sc}$, $AKIII_{Ec}$, and $HSK_{Sp}$

| Primer | Sequence |
|---|---|
| AKIIIEc-1 | 5′-CCCGAATTCCATATGTCT GAAATTGTTGTCTCC |
| AKIIIEc-2 | 5′-CCCAAGCTTCTCGAGTTAC TCAAACAAATTACTATGC |
| HSKSp-1 | 5′-GTCTCTAGAGCTAGCATGCAGA AATTTCAAATAAAAGTTCCAGC |
| HSKSp-2 | 5′-CGCGGATCCAAGCTTTCAAAAG TATTTGACTGTTGCACCGTC |

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.[17] 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 2, perhaps due to differences in their ACT domains which are responsible for their allosteric response to L-Thr and L-Lys, respectively.[18]

The lack of inhibition of $HSK_{Sp}$ may be explained by selection against a substrate analogue that resembles L-Asp rather than homoserine. The crystal structure of $HSK_{Mj}$ complexed with homoserine and an ATP analogue reveal that $HSK_{Mj}$-N141, which is equivalent to $HSK_{Sp}$-N135, interacts with the δ-OH of homoserine.[11] This side chain may be responsible for selecting against binding of L-Asp and for that matter our bisubstrate compounds.

## 3. 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 $sp^2$ versus. $sp^3$ 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.

## 4. Experimental

### 4.1. $AK_{Sc}$ cloning, expression, and purification

Cloning of the *HOM*3 gene, which encodes $AK_{Sc}$, has been previously reported.[19] 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 h. 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*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.

### 4.2. $AKIII_{Ec}$ cloning, expression, and purification

$AKIII_{Ec}$ was cloned using the PCR primers $AKIII_{Ec}$-1 and $AKIII_{Ec}$-2, Table 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*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 0–500 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.

### 4.3. *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 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*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.

### 4.4. 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.

### 4.5. 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.[20] 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.

### 4.6. Data Fitting

All data fitting performed used GraFit version 4 software.[21] 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).

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

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

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

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

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

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

### 4.7. 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*.[14] 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 ($2.2\times10^6$ 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.

### 4.8. 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 $2.2\times10^6$ 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 20×20 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.[22]

### 4.9. Preparation of compounds 1–4

General strategy: the appropriate dibromoalkane (2.6 equiv) was added to a stirred solution of Boc-Asp-OBzl (1 equiv) in 10 mL DMF at room temperature and stirred overnight.[23] The reaction was diluted with 100mL of water and the product extracted with ethylacetate (3×60 mL). The crude compounds were purified by column chromatography (Silica gel G60 A) using chloroform as the mobile phase.

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, $J_1=7.1$ Hz, $J_2=7.2$ Hz). *m*/*z* (relative intensity) 474 (M+2, 50%), 494.3 (M+Na, 100%). $R_f$ 0.92 ($CHCl_3$: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, $J_1=6.7$ Hz, $J_2=6.7$ Hz), 2.98 (m,2H), 1.80–1.89 (m,4H), 1.43 (s, 9H), 1.44–1.50 (m, 4H). *m*/*z* (relative intensity) 488 (M+2, 90%), 508.1 (M+Na, 10%). $R_f$ 0.98 ($CHCl_3$: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, $J_1=6.7$ Hz, $J_2=6.7$ Hz), 2.98 (m,2H), 1.79–1.93 (m,4H), 1.34–1.51 (s, 17H). *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, $J_1=6.7$ Hz, $J_2=6.7$ Hz), 2.98 (m,2H), 1.79–1.93 (m,4H), 1.34–1.51 (s, 19H). *m*/*z* (relative intensity) 516 (M+2, 8%), 559 (M+K, 10%). $R_f$ 0.95 ($CHCl_3$:MeOH:AcOH = 95:5:3).

### 4.10. Preparation of bisubstrate compounds 5–8 from 1–4

Separately compounds **1**–**4** (1 equiv) were added to a mixture of N-benzoyl-2′, 3′-*O*-isopropylideneadenosine **9** (1 equiv), tetrabutylammoniumbromide (2 equiv) and anhydrous DMSO (5 mL). KOH (2.5 equiv) 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 (3×30 mL).[24] 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 deprotection 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, $J=4.5$ Hz), 5.10–5.13 (d,1H, $J=6$ Hz), 3.37–3.43 (m,4H), 2.94 (m,2H), 1.46 (m,4H), 1.21–1.28 (t, 2H, $J_1=7.1$ Hz, $J_2=7.2$ Hz). *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, $J=4.5$ Hz), 4.08–4.20 (m,4H), 3.37–3.55 (m,4H), 2.94 (m,2H), 1.44–1.50 (m,4H). *m*/*z* (relative intensity) 480 (M-1, 35%). $R_f$ 0.42 ($CHCl_3$:MeOH = 9:1). (**7**) $^1$H NMR ($CDCl_3$) 8.78 (s, 1H), 8.21 (s,1H), 5.80 (d,1H, $J=4.5$ Hz), 4.08–4.20 (m,4H), 3.33–3.55 (m,4H), 2.94 (m,2H), 1.39–1.58 (m, 10H). *m*/*z* (relative intensity) 497 (M+1, 8%). $R_f$ 0.48 ($CHCl_3$:MeOH = 9:1). (**8**) $^1$H NMR ($CDCl_3$) 8.78 (s, 1H), 8.21 (s,1H), 5.80 (d,1H, $J=4.5$ Hz), 4.08–4.20 (m,4H), 3.33–3.70 (m,4H), 2.94 (m,2H), 1.39–1.58 (m,12H). *m*/*z* (relative intensity) 511 (M+1, 10%). $R_f$ 0.54 ($CHCl_3$:MeOH = 9:1).

### 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.

### References and notes

1. Viola, R. E. *Acc. Chem. Res.* **2001**, *34*, 339.
2. Angeles, T. S.; Hunsley, J. R.; Viola, R. E. *Biochemistry* **1992**, *31*, 799.
3. Stadtman, E. R.; Cohen, G. N.; LeBras, G.; de Robichon-Szulmajster, H. *J. Biol. Chem.* **1961**, *236*, 2033.
4. Ramos, C.; Delgado, M. A.; Calderon, I. L. *FEBS Lett.* **1991**, *278*, 123.
5. Keng, Y. F.; Viola, R. E. *Arch. Biochem. Biophys.* **1996**, *335*, 73.
6. Shaw, J. F.; Smith, W. G. *J. Biol. Chem.* **1977**, *252*, 5304.
7. Angeles, T. S.; Viola, R. E. *Arch. Biochem. Biophys.* **1990**, *283*, 96.
8. Huo, X.; Viola, R. E. *Biochemistry* **1996**, *35*, 16180.
9. Shames, S. L.; Wedler, F. C. *Arch. Biochem. Biophys.* **1984**, *235*, 359.
10. Zhou, T.; Daugherty, M.; Grishin, N. V.; Osterman, A. L.; Zhang, H. *Structure* **2000**, *8*, 1247.
11. Krishna, S. S.; Zhou, T.; Daugherty, M.; Osterman, A.; Zhang, H. *Biochemistry* **2001**, *40*, 10810.
12. Burr, B.; Walker, J.; Truffa-Bachi, P.; Cohen, G. N. *Eur. J. Biochem.* **1976**, *62*, 519.
13. Theze, J.; Kleidman, L.; St Girons, I. *J. Bacteriol.* **1974**, *118*, 577.
14. Wright, G. E.; Holman, T. R.; Walsh, C. T. *Biochemistry* **1993**, *32*, 5057.
15. Ogawa-Miyata, Y.; Kojima, H.; Sano, K. *Bioscience Biotechnology and Biochemistry* **2001**, *65*, 1149.
16. Ramos, C.; Calderon, I. L. *Applied and Environment Microbiology* **1992**, *58*, 1677.
17. Haouz, A.; Vanheusden, V.; Munier-Lehmann, H.; Froeyen, M.; Herdewijn, P.; Van Calenbergh, S.; Delarue, M. *J. Biol. Chem.* **2003**, *278*, 4963.

18. Aravind, L.; Koonin, E. V. *J. Mol. Biol.* **1999**, *287*, 1023.
19. Bareich, D. C.; Wright, G. D. *Biochem. Biophys. Res. Comm.* **2003**, *311*, 597.
20. Bareich, D. C.; Nazi, I.; Wright, G. D. *Chem. Biol.* **2003**, *10*, 967.
21. Leatherbarrow, R. J.; 4.0.14 ed.; Erithacus Software Ltd: Staines, UK, 2001.
22. Sillero, M. A.; Socorro, S.; Baptista, M. J.; Del Valle, M.; De Diego, A.; Sillero, A. *Eur. J. Biochem.* **2001**, *268*, 3605.
23. Bodansky, M. A. B. *The Practice of Peptide Synthesis*; Springer-Verlag: Bohn, Germany, 1984.
24. Liu, M.; Haddad, J.; Azucena, E.; Kotra, L. P.; Kirzhner, M.; Mobashery, S. *J. Org. Chem.* **2000**, *65*, 7422.