

STRUCTURE AND FUNCTION STUDIES OF MAMMALIAN
ADENOSINE KINASE

By

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STRUCTURE AND FUNCTION OF ADENOSINE KINASE

Abstract

Adenosine kinase (AK) is a purine salvage enzyme which catalyses the phosphorylation of the 5'-hydroxyl of adenosine via ATP. AK is a key enzyme which controls the intra and extracellular concentration of adenosine (Ado). Agents which inhibit the activity of AK have been found to attenuate cellular damage, demonstrating therapeutic utility in a variety of disease processes. In order to design inhibitors of AK with increased efficacy, a better understanding of enzyme activity is required.

Previously, a number of novel characteristics of mammalian AK had been discovered. It was shown that the activity of AK is influenced by the presence of pentavalent ions (PVI) such as inorganic phosphate (Pi), arsenate and vanadate. A detailed study of the influence of Pi on the kinetic parameters of Chinese hamster (CH) and beef liver AK was performed. These studies suggested that the K_m (Ado) decreases and the K_i (Ado) increases asymptotically in the presence of increasing concentrations of Pi. Under the same conditions, the V_{max} for activity increases hyperbolically. The effect of phosphate is not limited to the mammalian form of AK. Pi, arsenate and vanadate were all found to have similar effects on AK from yeast, spinach and *Leishmania donovani* AK. PVI as well as the metabolite phosphoenol pyruvate were also found to stimulate the activity of the enzyme ribokinase (RK) from *E. coli*, which similar to AK, is a member of the PfkB family of carbohydrate kinases.

Although AK and RK show little sequence similarity, the residues at the active site and the 3D structures of these two proteins are very similar. Based on sequence alignment of PfkB family members, we have identified a conserved sequence motif, NXXE, which based upon the available structural information appears to be involved in the binding of phosphate. To confirm and understand the role of this motif in Pi binding, the residues at the NXXE site were altered by site-directed mutagenesis and their effect on activity of the recombinant CH AK was examined. Though the residues at the NXXE site do not directly interact with substrate, nor the putative catalytic base, the resulting proteins were found to have greatly altered phosphate requirement, substrate inhibition characteristics and different magnesium requirements. In the AK structure, aspartic acid at position 316 is presumed to act as the catalytic base. This residue was changed to asparagine and glutamic acid by mutagenesis. The resulting proteins were found to be nearly completely devoid of activity, confirming its critical role in AK activity.

The amino acid sequence at the extreme N-terminus of AK has been found to exhibit the greatest variability within and among species, though the rest of the protein remains greatly conserved. To delineate the residues that are involved in the structural stability and activity of AK, systematic deletions of the residues from both the N- and C-terminus were performed, and the structure-activity relationships were examined. It was determined that the first 16 residues of CH AK can be removed without affecting activity. Removal of the next 11 residues resulted in sequential decreases in enzyme stability and activity. These 11 residues are involved in the first β -structure of the protein and are required for the stability of the tertiary structure. All residues at the C terminus were

required for activity, and involved in a hydrogen-bonding network necessary for the stability of the ATP binding site.

These studies provide novel insight to the structure-activity relationship of mammalian AK as well as the PfkB family of enzymes. Our work has identified a site distal to the catalytic site, that is implicated in the PVI binding and catalytic effect. Further studies should be aimed at understanding how binding of PVI at this site influences the catalytic activity of AK. Development of inhibitors which bind to this site and modulate the activity of AK should prove very useful in this regard.

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Preface

Portions of this thesis, Chapters 2, 3, 4, and 5 and parts of the Discussion (Chapter 6) contain figures and literature that have been published prior to the completion of this thesis. This preface draws attention to this fact in lieu of externally referencing the appropriate sections of the thesis.

Chapter 2 has been presented in the article entitled “The influence of inorganic phosphate on the activity of adenosine kinase” by M. Maj, B. Singh and R.S. Gupta (2000), *Biochim. Biophys. Acta.*, 1476 33-42;

Chapter 3 was published as “Structure-activity studies on mammalian adenosine kinase” by M.C. Maj, B. Singh and R.S. Gupta (2000), *Biochem. Biophys. Res. Commun.*, 275(2) 386-393;

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Table of Contents

TITLE PAGE	II
DESCRIPTIVE NOTE.....	III
ABSTRACT.....	IV
PREFACE.....	IX
FIGURE LEGEND.....	XV
TABLE LEGEND	XVII
ABBREVIATIONS	XVIII
CHAPTER 1. INTRODUCTION	1
1.1 ADENOSINE KINASE.....	2
<i>1.1.1 Activity.....</i>	<i>4</i>
1.1.1.1 Mechanism of Activity	4
1.1.1.2 Substrate Specificity and Inhibition	6
1.1.1.3 Pentavalent Ion Dependence.....	8
<i>1.1.2 Structure</i>	<i>10</i>
1.1.2.1 Primary Structure	10
1.1.2.2 Tertiary Structure	13
1.1.2.3 Motif 1	13
1.1.2.4 Motif 2	15
1.1.2.5 Ligand Binding.....	16
<i>1.1.3 Isolation and Characterization of Adenosine Kinase Mutants.....</i>	<i>18</i>
1.2 ADENOSINE	21
<i>1.2.1 Adenosine Metabolism</i>	<i>23</i>
<i>1.2.2 Adenosine Production</i>	<i>24</i>
1.2.2.1 Cytosolic-5'- Nucleotidase-1	24
1.2.2.2 Ecto-5'-nucleotidase.....	26
1.2.2.3 SAH.....	27
1.3 PURINE SALVAGE	28
1.4 ADENOSINE RECEPTORS.....	28
<i>1.4.1 A1 Receptors.....</i>	<i>32</i>
<i>1.4.2 A2A Receptors.....</i>	<i>32</i>
<i>1.4.3 A2B Receptors.....</i>	<i>33</i>
<i>1.4.4 A3 Receptors.....</i>	<i>33</i>
<i>1.4.5 Therapeutic Utilization of Adenosine Receptor Activation.....</i>	<i>34</i>
1.5 INHIBITORS OF ADENOSINE KINASE AS THERAPEUTIC AGENTS	34
1.6 PURPOSE OF THIS STUDY	37
1.7 REFERENCES.....	39

CHAPTER 2. THE INFLUENCE OF INORGANIC PHOSPHATE ON THE ACTIVITY OF ADENOSINE KINASE..... 56

2.1	PREFACE	57
2.2	PAPER	59
2.3	ABBREVIATIONS	60
2.4	ABSTRACT.....	61
2.5	INTRODUCTION.....	62
2.6	METHODS AND MATERIALS.....	64
2.6.1	<i>Materials</i>	64
2.6.2	<i>Radioactive Substrate Activity Assay</i>	64
2.6.3	<i>Data Analysis</i>	65
2.6.4	<i>Protein Purification</i>	66
2.6.5	<i>Structural Studies</i>	67
2.7	RESULTS.....	68
2.7.1	<i>Effect of Phosphate on Adenosine Affinity and Inhibition</i>	68
2.7.2	<i>Effect of Phosphate on ATP Affinity and Inhibition</i>	77
2.7.3	<i>ΔK_m & ΔK_i in the Presence of P_i</i>	80
2.7.4	<i>pH Studies</i>	80
2.7.5	<i>Structural Studies</i>	91
2.8	DISCUSSION.....	91
2.9	ACKNOWLEDGEMENTS.....	95
2.10	REFERENCES.....	96

CHAPTER 3. STRUCTURE-ACTIVITY STUDIES ON MAMMALIAN ADENOSINE KINASE 100

3.1	PREFACE	101
3.2	PAPER	103
3.3	ABBREVIATIONS	104
3.4	ABSTRACT.....	105
3.5	INTRODUCTION.....	106
3.6	METHODS AND MATERIALS.....	108
3.6.1	<i>Deletion and Site-directed Mutants of AK</i>	108
3.6.2	<i>AK Activity Assays</i>	109
3.6.3	<i>Structural Analysis</i>	110
3.7	RESULTS.....	110
3.7.1	<i>N-terminal Deletions</i>	111
3.7.2	<i>C-terminal Deletions</i>	117
3.7.3	<i>Active Site Mutants</i>	123
3.8	DISCUSSION.....	126
3.9	REFERENCES.....	132
3.10	APPENDIX.....	136

CHAPTER 4. THE EFFECT OF INORGANIC PHOSPHATE ON THE ACTIVITY OF BACTERIAL RIBOKINASE..... 138

4.1	PREFACE	139
4.2	PAPER	141
4.3	ABBREVIATIONS	142
4.4	ABSTRACT.....	143
4.5	INTRODUCTION.....	144
4.6	METHODS AND MATERIALS.....	146
4.6.1	<i>Materials</i>	146
4.6.2	<i>Activity Assay</i>	146
4.7	RESULTS.....	147
4.7.1	<i>Development of the Stopped Radioactive Assay</i>	147
4.7.2	<i>Effects of Pentavalent ions on Activity and Km D-ribose</i>	148
4.7.3	<i>Effects of Phosphoenolpyruvate on Activity and Affinity for D-ribose</i> ...	151
4.7.4	<i>Effects of Pentavalent Ions on Activity and Substrate Affinity for ATP</i> ..	151
4.8	DISCUSSION.....	157
4.9	ACKNOWLEDGMENTS.....	160
4.10	REFERENCES.....	161
4.11	APPENDIX A	164

CHAPTER 5. PENTAVALENT ION DEPENDENCY IS A CONSERVED PROPERTY OF ADENOSINE KINASE FROM DIVERGENT SOURCES: IDENTIFICATION OF A NOVEL MOTIF IMPLICATED IN PHOSPHATE AND MAGNESIUM ION BINDING AND SUBSTRATE INHIBITION. 166

5.1	PREFACE	167
5.2	PAPER	169
5.3	ABBREVIATIONS:	170
5.4	ABSTRACT.....	171
5.5	INTRODUCTION.....	173
5.6	METHODS AND MATERIALS.....	180
5.6.1	<i>Cell Extracts</i>	180
5.6.2	<i>Site-Directed Mutants of Adenosine Kinase</i>	181
5.6.3	<i>Adenosine Kinase Activity Assays</i>	182
5.6.4	<i>Molecular Modeling</i>	183
5.7	RESULTS.....	183
5.7.1	<i>Effects of Pi on the Initial Velocity of AK from Diverse Sources</i>	184
5.7.2	<i>Effects of Pi on Site-directed Mutants Altered in the NXXE Motif</i>	188
5.7.3	<i>Mg²⁺ Ion Dependency of the WT AK and Various Mutants Affected in the NXXE Motif</i>	195
5.8	DISCUSSION.....	200
5.9	ACKNOWLEDGMENTS.....	208
5.10	REFERENCES.....	209
5.11	APPENDIX.....	216

CHAPTER 6. DISCUSSION AND CONCLUSIONS.....	220
6.1 DISCUSSION AND CONCLUSIONS.....	221
6.2 FUTURE DIRECTIONS.....	227
6.3 REFERENCES.....	230

Figure Legend

Figure 1.1 Structure of Adenosine	7
Figure 1.2 Sequence Alignment of Adenosine Kinase and Bacterial Ribokinase	12
Figure 1.3 Structures of AK from <i>T. gondii</i> and RK from <i>E. coli</i>	14
Figure 1.4 Unusual O4'-endo Puckering of Adenosine.	17
Figure 1.5 Examples of N- and C-nucleosides.	20
Figure 1.6 Adenosine Production in the Cell.....	25
Figure 1.7 <i>De Novo</i> Pathway of AMP Synthesis.....	29
Figure 1.8 Purine Salvage from S-adenosylmethionine to AMP.....	30
Figure 1.9 The Structure of AK Inhibitors.	36
Figure 2.1 Effect of [Pi] on CHO AK K_m & K_i for adenosine at pH 6.2.	71
Figure 2.2 Effect of [Pi] on BL AK K_m & K_i for adenosine at pH 6.2.....	73
Figure 2.3 Effect of [Pi] on CHO AK and BL K_m & K_i for adenosine at pH 7.4.	75
Figure 2.4 Effect of [Pi] on CHO and BL AK K_m^{ATP} at pH 6.2.....	79
Figure 2.5 Summary of Observed Affinity Constants of AK for Adenosine.....	82
Figure 2.6 Summary of Observed Substrate Inhibition Constants of AK.	84
Figure 2.7 Effect of Phosphate on pH Optimum.	86
Figure 2.8 CD Spectra of CHO AK	88
Figure 2.9 Fluorescence Emission Spectrum of CHO AK.....	90
Figure 3.1 Sequence Alignment of AK.....	113
Figure 3.2 Effect of N-terminal Deletions (A) on AK Activity and Biochemical Characteristics (B).....	116
Figure 3.3 A Ribbon Diagram Depicting the N-terminal Region of AK.....	119
Figure 3.4 Effect of C-terminal Deletions (A) on AK Activity and K_m (B).....	122
Figure 3.5 Ribbon Diagram for the C-terminal Region of AK.....	125
Figure 3.6 Proposed Catalytic Role of the D316 Residue.....	128
Figure 3.7 Thermal inactivation kinetics.....	137
Figure 4.1 The Effect of Pentavalent Ions on K_m D-ribose.	149
Figure 4.2 Effect of Pentavalent Ions on the Maximum Velocity.	153
Figure 4.3 The Effect of Pentavalent Ions on K_m ATP.....	155
Figure 4.4 Ribbon Diagram of RK and AK.....	164

Figure 5.1 The Active-Site Structure of Adenosine Kinase and Ribokinase.	176
Figure 5.2 Sequence Alignment of Representative Proteins from the PfkB Family of Carbohydrate Kinases.	178
Figure 5.3 Effects of Pentavalent Ions on the Initial Velocity and K_m for Adenosine. ...	191
Figure 5.4 Effects of Pentavalent Ions on the Initial Velocity and K_m for ATP.	193
Figure 5.5 Magnesium ion dependencies of WT and various mutant AK	197
Figure 5.6 A Model Showing the Interactions of Substrates and Ligands at the Active Site of Adenosine Kinase.	205
Figure 5.7 Effect of Pentavalent Ions on the Activity and K_m for Adenosine for Various AK from Cell Extracts	216
Figure 5.8 Effect of Pentavalent Ions on the Activity and K_m for ATP for Various AK from Cell Extracts.	218
Figure 6.1 The Adenosine Binding site of AK.	229

Table Legend

Table 1.1 K_m and pH Optimum for AK from a Variety of Sources	9
Table 2.1 Effect of [Pi] on the Kinetic Constants of AK for Adenosine at pH 6.2.	76
Table 2.2 Effect of [Pi] on the Kinetic Constants of AK for Adenosine at pH 7.4.	76
Table 2.3 Effect of Phosphate on K_m^{ATP} at pH 6.2.....	76
Table 4.1 Effect of Pentavalent Ions on the Activity and K_m of Ribokinase for	152
Table 5.1 Steady-State Kinetic Parameters of Co-substrate Adenosine for AK in Cell Extracts	186
Table 5.2 Steady-State Kinetic Parameters of Co-substrate ATP for AK in Cell Extracts	187
Table 5.3 Steady-State Kinetic Parameters of Co-substrate Adenosine for Wild-type and Site-directed Mutants.....	190

Abbreviations

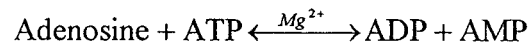
5IT, 5-iodotubercidin
AICAR, phosphoribosyl aminoimidazole carboxyamide
AIR, phosphoribosyl aminoimidazole
AD, adenosine deaminase
Ado, adenosine;
AK, adenosine kinase;
Arg, arginine
Asn, asparagine
Asp, aspartic acid
APRT, adenine phosphoribosyl transferase
BL, beef liver
CH, Chinese hamster
CHO, Chinese hamster ovary;
cN-1, cytosolic-5'-AMP-nucleotidase
D, aspartic acid
E, glutamic acid
e-N, ecto-5'-nucleotidase
FGAM, phosphoribosylformyl glycinamide
GAR, glycinamide ribonucleotide
Gln, glutamine
Gln-PRPP, glutamine phosphoribosyl pyrophosphate
Glu, glutamic acid
MCAO, middle cerebral artery occlusion
 Mg^{2+} , free magnesium ion
 MO_6 , octahedral coordination of free magnesium
MTA, methylthioadenosine
N, asparagine
NXXE, novel motif of PfkB kinases
P1, purinergic receptors
PEP, phosphoenolpyruvate;
PfkB, classification of a family of carbohydrate kinases which sequence show homology to the minor isoform of phosphofructokinase, or phosphofructokinase B
PPRP, 5-phospho- α -D-ribose 1-diphosphate
Pi, inorganic phosphate;
PVI, pentavalent ions;
Q, glutamine

R, arginine
R-5-P, D-ribose 5-phosphate;
RK, ribokinase;
SAH, S-adenosylhomocysteine
SAICAR, phosphoribosyl aminoimidazole succinocarboxamide
SAME, S-adenosylmethionine
TG, *Toxoplasma gondii*
WT, wild-type Chinese hamster ovary adenosine kinase

Chapter 1. Introduction

1.1 Adenosine Kinase

Adenosine kinase (AK: ATP:adenosine 5'-phosphotransferase, EC 2.7.1.20) is a cytosolic, single polypeptide purine salvage enzyme which is exclusive to eukaryotes. AK catalyzes the phosphorylation of the 5' hydroxyl of adenosine (co-substrate 1) to form AMP via ATP (co-substrate 2) according to the reaction:



The structural and kinetic properties of AK distinguish it among all other known nucleoside kinases which have been characterized to date. In contrast to other nucleoside kinases, AK behaves as a monomer, with lower specificity for deoxynucleosides [1], and does not contain typical nucleotide binding motifs [2].

AK was first discovered in yeast extracts by Caputto in 1951 [3] and later in mammalian tissues [4]. AK is the most abundant nucleoside kinase known. It has been found in every mammalian tissue studied and has been isolated from a number of eukaryotic microorganisms and plant species [2;5-26]. The purine salvage pathway involves the synthesis of purine nucleotides from pre-formed purine bases, the product of nucleotide degradation. The salvage pathway of purine biosynthesis is of greater energy efficiency than the *de novo* pathway which involves multiple successive steps by highly regulated enzymes [27]. The purine salvage pathway has been adopted by parasitic

organisms, such as *Leishmania donovani* and *Toxoplasma gondii*, which do not contain *de novo* mechanisms of purine biosynthesis.

AK plays an important role in the regulation of intracellular levels of adenosine [28;29]. The steady-state concentrations of adenosine are kept in the nanomolar range by AK and adenosine deaminase (AD). AK has a substantially lower *K_m* for adenosine than that of adenosine deaminase and, as such, is thought to have principle involvement in adenosine metabolism [15;29;30]. Much recent interest in adenosine metabolism has arisen from the discovery that adenosine, through interaction with adenosine receptors, is involved in the protection of cells undergoing stress and trauma. The formation of adenosine is accelerated under conditions of tissue stress. Inhibition of AK has been proven to substantially augment this local increase in adenosine concentration thereby enhancing the therapeutic activity of adenosine. AK inhibitors, based on the structure of adenosine, have been shown to have potent therapeutic utility in a variety of disease processes such as ischemia, inflammation, pain and epilepsy. A number of adenosine analogs can also be phosphorylated by AK [31]. This activity is currently being exploited in the field of chemotherapy [32;33], as the phosphorylated form of a number of these purine nucleoside analogues show cytotoxic and antiviral properties [18;34-36].

As mentioned previously, AK bears little sequence similarity to other nucleoside kinases, but shares two motifs of high similarity with other carbohydrate kinases classified as belonging to the ribokinase family or PfkB family, which is termed for the minor isoform of 6-phosphofructokinase. This suggests an evolutionary connection for AK to microbial ribokinases and fructokinases. AK is monomeric and is composed of two

domains. The large domain has the overall structure of a 3-layer $\alpha\beta\alpha$ sandwich. The smaller domain acts as a lid over the active site. The active site lies in a cleft between the large and small domain [37].

1.1.1 Activity

1.1.1.1 Mechanism of Activity

Despite a large number of studies, the mechanism of AK activity and substrate binding properties are poorly understood. The activity of AK can become inhibited by high concentrations of both co-substrates as well as high concentrations of free magnesium (Mg^{2+}). Further complications involve the recent discovery that pentavalent ions (PVI) stimulate the activity of AK [38]. While it is intriguing that the enzyme is tightly regulated by substrate concentrations, it is also the cause of difficulties in the interpretation of kinetic data. This has resulted in great controversy regarding the mechanism of activity.

Biochemical studies have been performed on purified AK from a number of sources including yeast [6], *Leishmania donovani* [39], rat liver and brain [40;41], murine leukemia L1210 cells [42], rabbit liver [35], and human liver, placenta, and erythrocytes [12;43]. Most studies suggest a sequential kinetic mechanism, however differ in the order of substrate binding and product release. The majority of studies suggest that adenosine is the first substrate to bind and AMP the last product to be released [31;44-46]. Other studies show ATP binding first and either AMP [19;47] or ADP as the last product to leave [40]. One study with AK from murine leukemia L1210 cells reports a ping-pong mechanism which requires a phosphorylated enzyme intermediate [42]. AK from rat liver [48], Syrian

hamster and bovine liver [49] have been shown to catalyze an exchange reaction between adenosine and AMP in the presence of ADP, and absence of ATP, supporting a ping-pong mechanism. However, a phosphoryl-enzyme species has yet to be discovered [40;42;49], and vanadate, which is a potent inhibitor of phosphorylated enzyme intermediates, does not inhibit the activity of AK [31;49]. Mechanistic studies with $(\gamma\text{R})\text{ATP}\gamma\text{S},\gamma^{18}\text{O}$ have shown that phosphate transfer occurs directly with an inversion of configuration for the γ -phosphate of ATP suggesting an $\text{S}_{\text{N}}2$ type in-line mechanism [50]. Therefore, it is generally accepted that the mechanism of AK activity follows an ordered bi-bi mechanism.

The determination of optimal conditions to assay AK activity remains unclear. Various optimal assay conditions have been reported, revealing a complex relationship between pH and activity. Considerable documentation shows that the most favorable pH for activity is dependant upon the $\text{ATP}^{4-}/\text{Mg}^{2+}$ ratio [14;39;44;51;52]. Optimum activity also varies with the ratio of Mg^{2+} to adenosine [14;39;53]. Studies with AK from human erythrocytes have shown that pH optimum was 5.1 with $\text{Mg}^{2+}/\text{ATP}^{4-}$ ratio of 5.0 in sodium acetate buffer, and pH 6.2 with ratio at 0.5 in PIPES buffer [16]. AK from Swiss-Webster mice thymus tissue require $\text{Mg}^{2+}/\text{ATP}^{4-}$ ratio of 1:5 in Tris-maleate buffer at pH 6.0 [54]. It has also been reported for rat brain AK, that pH optima is biphasic at low adenosine concentrations (sharp optima at pH 5.5 accompanied by a broad optima from pH 7.5 to 8.5), where 25 μM adenosine shifts the profile to monophasic, peaking at pH 6.0 [41]. Further, the choice of buffer can shift maximal activity as much as 1 pH unit [14;51;55]. Whether the buffer influences substrate affinity or inhibition is not known.

1.1.1.2 Substrate Specificity and Inhibition

AK has a broad substrate specificity. A number of nucleoside anti-viral, anti-coccidial and anti-cancer drugs are active only upon phosphorylation by AK at the 5' hydroxyl of the sugar base [14;56;57]. Ribavirin, which is activated by AK [58], has recently been shown exert its anti-viral effect by directly incorporating into viral RNA [59]. Classic AK substrates with broad spectrum biological activities are 7-deazaderivatives of adenosine which include Tubercidin, the iodinated derivative iodotubercidin, and the nitrile derivative toyocamycin, as reviewed by Gupta [34] and others [60-63]. The substitution of carbon with nitrogen at position 8, or 8-azaadenosine, is also efficiently phosphorylated by AK [35]. So too are nucleosides which differ from adenosine (Fig. 1.1) at atom 2 and 6 of adenosine [35;51].

Alteration of the ribosyl moiety decreases substrate efficiencies. 2'-deoxyadenosine has been shown as a poor substrate [35]. It has been shown that the 2'-hydroxyl must be in the *trans* position relative to the purine base allowing for freedom of rotation between the C-1* and N-9 bond [64]. The 3' hydroxyl and 4'-hydroxy-methyl can be tolerated in either in the *cis* or *trans* conformation [64]. Recent crystallographic data has revealed that ribose will adopt an unusual endo pucker upon binding to adenosine kinase and ribokinase (*rf.* section 1.2.2.4 Fig. 1.4)

ATP is the preferred phosphate donor for mammalian AK, however GTP can substitute in the catalytic reaction [12;19;35;51;65]. Chicken liver AK has been found to utilize a broader number of triphosphate donors than AK from protozoan parasites of the *Eimeria* genus, the order of triphosphate donors being ATP > dATP > GTP > dGTP > ITP

[66]. Mg^{2+} is required for activity, with Mg/ATP^{2-} being the true substrate [13;39;51;67].

The mechanism by which substrate inhibition occurs for AK remains unclear. Strong substrate inhibition at high concentrations of adenosine [14;18;44;53] has been shown for AK from every source except for purine auxotrophs, such as the parasitic organism *Leishmania donovani* [39]. It has been proposed that AK has two adenosine binding sites, that of a catalytic or 'high affinity' site and a regulatory 'low affinity' site [45]. Protein fluorescence quenching studies failed to reveal the presence of a 'regulatory' adenosine binding site, however these studies did show that both adenosine and AMP will

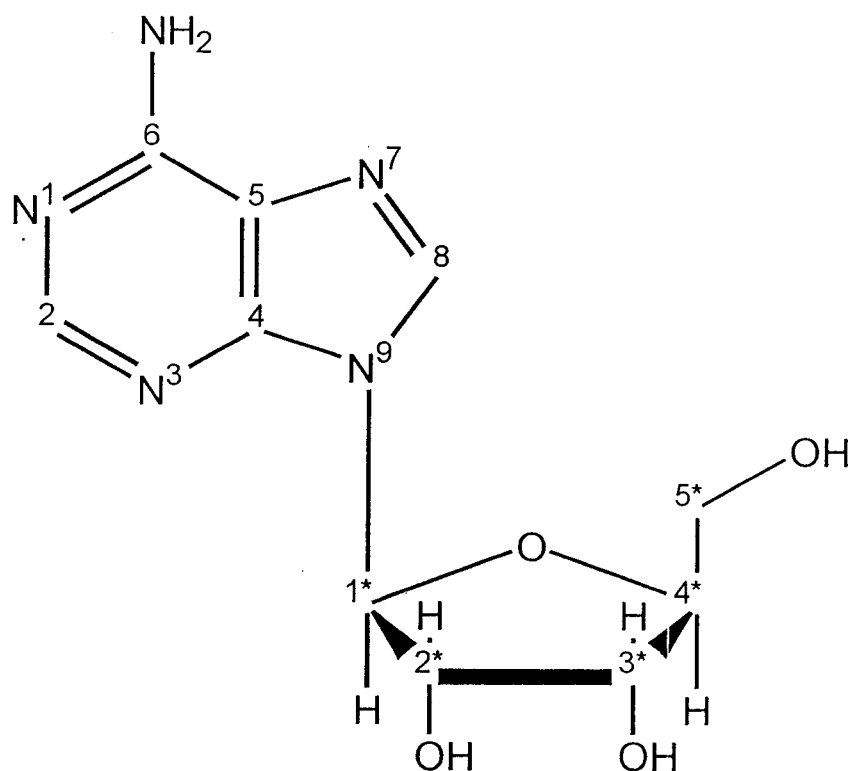


Figure 1.1 Structure of Adenosine

The structure of adenosine (6-amino-9-β-D-ribofuranosyl-9-H-purine) with atom numbering which follows IUPAC/IUB conventions.

bind with low affinity to the ATP site. These studies also indicate that ATP and ADP will not bind at the adenosine site [68]. Because of the structural similarities between co-substrates, it has been suggested that the adenosine binding at the ATP binding site may be responsible for substrate inhibition [68;69].

High concentrations of free Mg^{2+} [18;44;65] and Mg^{2+}/ATP^{4-} [39;70] are also known to inhibit AK. The degree of inhibition by adenosine and Mg^{2+}/ATP^{4-} are affected by the concentration of free Mg^{2+} [14;67] and are interconnected with the pH [12]. Thus, sub-saturating levels of co-substrates and Mg^{2+} are often used when assaying for activity, complicating kinetic studies, and K_m determination are typically apparent values. Moreover, these K_m values are determined under a variety of conditions by various workers, where optimum ratios of co-substrates and Mg^{2+} are shown to fluctuate with the pH of the assay. Therefore, it is difficult to compare the activities of AK from different sources in the absence of a defined set of experimental conditions. The K_m for adenosine ranges from 0.041 to 33 μM and K_m for ATP from 0.02 to 600 μM for Mg^{2+}/ATP^{4-} (Table 1.1).

1.1.1.3 Pentavalent Ion Dependence

In 1996, a novel characteristic of AK was revealed, which showed that activity exhibits a dependence upon pentavalent ions such as phosphate (Pi), arsenate, and vanadate. The activity of AK purified from Syrian hamster and bovine liver was stimulated upon the addition of these pentavalent ions, and the affinity of the enzymes for adenosine was increased, without affecting the affinity for ATP [38]. Similarly, the exchange reaction

Table 1.1 *K_m* and pH Optimum for AK from a Variety of Sources

Source of enzyme	pH optima	pH of assay	<i>K_m</i> Adenosine μM	<i>K_m</i> ATP μM	Ref.
<i>Tissue</i>					
Human placenta	5.5-6.5	5.5	0.4	75	[12;44]
Human liver	5.5 & 7.5-8.5	7.5	0.15	73	[18]
Human erythrocyte		7.3	0.66	82	[16]
H.Ep.No.2 cells	6.2-6.8	7.0	1.8		[71]
Erlich ascites tumor cells		5.0	5.8		[51]
Murine leukemia L1210		6.8	1.7		[52]
Rat brain	5.5 & 7.5-8.5	7.5	0.2	0.02	[41]
Rat heart		7.0	0.2	0.8	[67]
Guinea pig brain		5.8	20		[7]
Bovine adrenal medulla		7.5	0.2	20	[31]
Rabbit liver	4.8-5.5	6.8	0.4	600	[14;35]
		5.0	1.6		[51]
<i>T. gondii</i>		7.5	1.9	54.4	[72]
<i>L. donovani</i>	7.5-8.5	7.5	33	50	[39]
		7.5	16		[73]
<i>Recombinant</i>					
<i>Arabidopsis</i> ADK 1		7.2	0.50	350	[74]
<i>Arabidopsis</i> ADK 2		7.2	0.30	370	[74]
Human		7.0	0.041		[2]
Human AK short		7.5	0.12	~250	[21]
Human AK long		7.5	0.13	~250	[21]

also showed a dependence on pentavalent ions [49]. A number of other cations and anions were investigated for both the forward and reverse reactions (K^+ , Na^+ , Fe^{2+} , Cu^{2+} , Ca^{2+} , Zn^{2+} , acetate, sulfate, sulfite, nitrate, carbonate). Up to a concentration of 20 mM, only pentavalent ions were found to stimulate activity [38;49]. The requirement of inorganic phosphate has not previously been reported, however commercial preparations of ATP are generally contaminated with 1-2% inorganic phosphate [75]. Therefore, all studies of AK activity to date have likely been performed in the presence of inorganic phosphate.

Two other enzymes have shown similar pentavalent ion dependence, 6-phosphofructo-2-kinase (EC 2.7.1.105) and phosphoribosylpyrophosphate synthetase (EC 2.7.6.1) [75;76]. These enzymes share the feature of phosphate transfer from ATP to a sugar derivative, or co-substrate 1. It has been shown for AK that phosphate is not consumed or exchanged with the reactants; nor is a phosphate intermediate formed [49]. It is thought that phosphate may induce an active protein conformation, thereby facilitating the binding of co-substrate 1.

1.1.2 Structure

1.1.2.1 Primary Structure

AK is a monomeric protein with a molecular weight from 38-50 KDa, depending upon the enzyme source. In 1994, the first amino acid sequence of AK from *Leishmania donovani*, was directly submitted to the EMBL Data Library by Mirsra, Banyopadhyay and Datta, accession number S52758. The following year, cDNA from a variety of mammalian sources, bearing absolutely no resemblance to the cDNA from the

Leishmania source, was directly deposited to the same data library by Gupta and Singh. At the time, there were no reports as to an active protein being expressed from the above cDNA sequences. The incongruity of the primary structure was resolved in 1996 with the first report of functional recombinant AK from a human liver cDNA library, which closely resembled the cDNA reported by Gupta and Singh [2]. The sequence encoding for *Leishmania donovani* AK was later corrected in 1998, revealing 42% identity to the mammalian enzymes. Examples of active recombinant AK now include the parasitic organism *Toxoplasma gondii* [72], the moss *Physcomitrella patens* [22], two isoforms of human AK [21], and two isoforms of *Arabidopsis thaliana* [74]. One isoform of *Arabidopsis*, termed ADK2, behaves similarly to mammalian enzymes, and ADK1 shows a much greater tolerance for high concentrations of adenosine though the sequences are 92% identical [74].

Primary sequence information has revealed that AK belongs to an evolutionarily related family of carbohydrate kinases termed PfkB [77;78]. The sequence of family members do not contain the classic N-terminal P-loop motif [2], common to many ATP and GTP-binding proteins [79]. Other members of this family include inosine-guanosine kinase (EC 2.7.1.73), fructokinase (EC 2.7.1.4), 1-phosphofructokinase (EC 2.7.1.56), 2-dehydro-3-deoxyglucokinase (EC 2.7.1.45) and 6-phosphofructokinase minor (EC 2.7.1.11). Recent additions to the family include pyridoxal kinase (EC 2.7.1.35) and phosphomethylpyrimidine kinase (EC 2.7.4.7). The PfkB family is identified by two

CHO	MAAAEE- PKP	KKLKVEAPQA	LSENVLFGMG	NPLLDISAVV	DKDFLDKYSL	KPNDQILAE	K----	HKELF	65
HUM L	MAAAEEPKP	KKLKVEAPQA	LRENILFGMG	NPLLDISAVV	DKDFLDKYSL	KPNDQILAED	K----	HKELF	66
HUM S	MTSV-----	-----	-----	-----	-----	-----	-----	-----	49
AT	MASSSN----	-----	YDGILLGMG	NPLLDISAVV	DDEFLTKYDI	KLNNAILAED	K----	HLPY	51
LD	M-----	-----	SALPQLYIQ	NPLLDVSAVP	DDAFLEKYKV	QKTSACLMEE	I----	HKGIF	47
SC	M-----	-----	---TAPLVVLG	NPLLDFOADV	TAEYLAKYSL	KENDAILVDA	KSGDARMAIF	49	
TG	MAVDSS----	-----NSA	TGPMRVFAIG	NPILDVLAEV	PSSFLDEFFL	KRGDATLATP	EQ----	MRIY	55
RK	MQNA-----	-----	---GSLVVLG	SINADHILNL	QSFPTPG---	-----	-----	-----	28
CHO	DELVRKFKVE	YHAGGSTQNS	IKVAQWMIQK	PH-KAATFFG	CIGIDKFGEI	LKSKAAEAHV	DAH-YYEQNE	133	
HUM LG	DELVKKFKVE	YHAGGSTQNS	IKVAQWMIQQ	PH-KAATFFG	CIGIDKFGEI	LKRKAAEAHV	DAH-YYEQNE	134	
HUM SH	DELVKKFKVE	YHAGGSTQNS	IKVAQWMIQQ	PH-KAATFFG	CIGIDKFGEI	LKRKAAEAHV	DAH-YYEQNE	117	
AT	DEMSSKFNVE	YIAGGATQNS	IKVAQWMLQI	P--GATSYMG	SIGKDKYGEA	MKKDATAAGV	NVH-YYEDES	118	
LD	EELEQHPNVT	YVEGGSGLNT	ARVAQWIAQA	PKSSLFNIVG	CASDDRYGKI	LKEAAEKNGV	NMH-LEYTTK	116	
SC	DELLQMPETK	LVAGGAAQNT	ARGAAYVLAG	GQV---VYFG	SVGKDKFSER	LLNENEKAGV	KSM-YQVQND	115	
TG	STLD-QFNPT	SLEGGSSALNS	VRVVQKLLR-	KP-GSAGYMG	AIGDDPRGQV	LKELCDKEGL	ATR-FMVQAD	102	
RK	-ETVTGNHYQ	VAFGGKGANQ	AVAAGRSGA-	----NIAFIA	CTGDDSIGES	VRQQLATDNI	DITPVSVIKG	92	
CHO	QPTGTCAACI	TG-DNRSLVA	NLAAANCYKK	EKHLDLLENW	VLVEKARVYY	IAGFFLTVSP	ESVLKVARYA	202	
HUM LG	QPTGTCAACI	TG-DNRSLIA	NLAAANCYKK	EKHLDLLEKNW	MLVEKARVCY	IAGFFLTVSP	ESVLKVAHHA	203	
HUM SH	QPTGTCAACI	TG-DNRSLIA	NLAAANCYKK	EKHLDLLEKNW	MLVEKARVCY	IAGFFLTVSP	ESVLKVAHHA	186	
AT	APTGTGCVCV	VG-GERSLIA	NLSAANCYKV	D-HLKKPENW	ALVEKAKFYY	IAGFFLTVSP	ESIQLVSEHA	186	
LD	APTGSCAVCI	SG-KDRSLVA	NLSAANLLSA	D-HMSSSDV	ETLKGCCQLY	LTGFTLTIDV	NYVLQVAAEA	184	
SC	IGTGKCAALI	TG-HNRSLVT	DLGAANFFTP	D-HLDC-HWD	LV-EAAKLFY	IGGFHLTVSP	DAIVKLGQHA	181	
TG	QSTGTCAVLI	NE-KERTLCT	HLGACGSFRI	---PED---WT	TFASGALIFY	ATAYTLTATP	KNALEVAGYA	155	
RK	ESTGVALIFV	NGEGENVIGI	HAGANAALSP	ALV---EAQR	ERIANASALL	MQLESPL---	ESVMAAKIA	156	
CHO	AENNR-IFTL	NLSAPFISQF	FKESLME-VM	PYVDILFGNE	TEAATFAREQ	GFETKDIKEI	AKKAQALAKV	270	
HUM LG	SENNR-IFTL	NLSAPFISQF	YKESLMK-VM	PYVDILFGNE	TEAATFAREQ	GFETKDIKEI	AKKTQALPKM	271	
HUM SH	SENNR-IFTL	NLSAPFISQF	YKESLMK-VM	PYVDILFGNE	TEAATFAREQ	GFETKDIKEI	AKKTQALPKM	254	
AT	AANNK-VFTM	NLSAPFICEF	FKDVQEK-FL	PYMDVVFQNE	TEARTFSRVH	GWETEDVEQI	AIRKISQLPKA	254	
LD	RASGG-QFMM	NLSAPFVLQY	FTESFNK-AA	PYLDVIFGNE	VEAKALADAM	KWNPASTHNL	AKKAAMELPY	252	
SC	KENSK-PFVL	NFSAPFIPHV	FKDALAR-VL	PYATVIANE	SEAEAFCDAF	QLDCANTDLE	AIAQRIVKDS	249	
TG	HGIPNAIFTL	NLSAPFCVEL	YKDAMQS-LL	LHTNILFGNE	EEFAHLAKVH	NLVAADKTAL	STANKEHAVE	254	
RK	HQNKI-IVAL	NPAPA-RE--	----LPDELL	ALVDIITPNE	TEAEKLTGIR	VENDEDAAKA	AQVLHEKG--	216	
CHO	NS-----	----KRPR	VVFTQGRD	VVATE-----	NEVMAFAVLD	QNO-KEIIDT	NGAGDAFVGG	321	
HUM LG	NS-----	----KRQRI	VI FTQGRD	IMATE-----	SEVTAFVLD	QDQ-KEIIDT	NGAGDAFVGG	322	
HUM SH	NS-----	----KRQRI	VI FTQGRD	IMATE-----	SEVTAFVLD	QDQ-KEIIDT	NGAGDAFVGG	305	
AT	TG-----	----TYKRT	TVITQGD	VVAED-----	-GKVKYPVI	PLPKEKLVDT	NGAGDAFVGG	305	
LD	SG-----	----TRDRI	VDFTQGSQPT	VYATR-----	SGKTGSVTQV	PIAHDIIVDL	NGAGDAFVGG	304	
SC	PV-----	----EKTVI	FTHGVEPTV	VSSKG-----	---TSTYPVK	PLDSSKIVDT	NGAGDAFVGG	298	
TG	VCTGALRLLT	AGQNTGATKL	VVMTRGHNPV	IAAEQTADGT	VVVHEVGVP-	VVAAEKIVDT	NGAGDAFVGG	323	
RK	-----	-----IRT	VLITLGRGV	WASVN-----	GEGQRPVGF	-VQ---AVDT	IAAGDTFNGA	260	
CHO	FLSQLVYNKP	LTECIRAGHY	AASVIIRRTG	-CTFPEKPDF	H			361	
HUM LG	FLSQLVSDKP	LTECIRAGHY	AASIIIRRTG	-CTFPEKPDF	H			362	
HUM SH	FLSQLVSDKP	LTECIRAGHY	AASIIIRRTG	-CTFPEKPDF	H			345	
AT	FMSQLVKEKS	IEECVKAGCY	ASNVIQRSG	-CTYPEKPDF	N			345	
LD	FLAAYAMSCS	IQRCEVGNY	AAGVIOHNG	-CTYPEKPSI	SP			345	
SC	FMAGLTKGED	LETSIDMGQW	LAALSIQEVG	-PSYPSEKIS	YSK			340	
TG	FLYGLSQGKT	VKQCIMCGNA	CAQVDIQHVG	-FSLSFTSLP	C			363	
RK	LITALLEEKP	LPEAIRFAHA	AAAIATVRKG	AQPSVPWREE	IDAFLDQR			309	

Figure 1.2 Sequence Alignment of Adenosine Kinase and Bacterial Ribokinase.

AK sequences are compared to RK. The annotations are: **CHO**, Chinese hamster AK P55262; **HUM LG**, human AK long isoform AAB50234.1; **HUM SH**, human short isoform AAB50235.1; **AT**, *A. thaliana* AK isoform AAK68795.1; **LD**, *L. donovani* AK AAC80288.1; **SC**, *S. cerevisiae* NP_012639.1; **TG**, *T. gondii* AK 1DH2A; and **RK**, *E. coli* ribokinase P05054. A structural alignment for HUM ST, TG and RK performed using Swiss-PdbViewer version 3.6b2, the rest of AK sequences were then aligned with ClustalW. PfkB sequence motif 1 is outlined in red, motif 2 in blue, and the conserved NETE motif in green. The residues highlighted by red circles are involved in H-bonding to stabilize domain closure. Putative catalytic base is indicated by the blue triangle.

consensus patterns. Motif 1 is found near the N-terminus, [AG]-G-X(0,1)-[GAP]-X-N-X(6)-[GS]-X(9)-G, and motif 2, [DNSK]-[PSTV]-X-[SAG](2)-[GD]-D-X-X-X-[SAGV]-[AG]-[LIVMFYA]-[LIVMSTAP] is found near the C-terminus (Fig. 1.2).

1.1.2.2 Tertiary Structure

The crystal structure of 2 PfkB family members have recently been solved, AK and ribokinase (RK) [37,80-82]. The primary sequence of AK and RK has approximately 25% sequence homology, and AK is monomeric whereas RK behaves as a dimer. Remarkably, the 3D structures are superimposable (Fig 1.3). Both proteins are comprised of a large and small domain, with the domains connected by 4 peptide segments. The interior face of the small domain acts as a lid over the active site, which is located in the cleft between the small and large domains. The large domain is comprised of a 3 layer $\alpha\beta\alpha$ sandwich structure. The most visible difference between the two structures is seen in the small domain. Dimer formation of RK occurs via β -sheet interaction of the outer face of the small domain referred to as a ' β -clasp' structure which resembles a hand-shake [81]. The surface of the AK small domain contains 2 helix structures not seen in the RK structure, which do not allow for dimer formation.

1.1.2.3 Motif 1

The 3D structures of *Toxoplasma gondii* (TG) AK and bacterial RK, bound with various ligands, indicate that the conserved motif 1 forms the first helical structure of the large domain which forms direct interactions with adenosine and ribose respectively. Motif 1 also contains a 'GG' switch region that functions as a hinge for

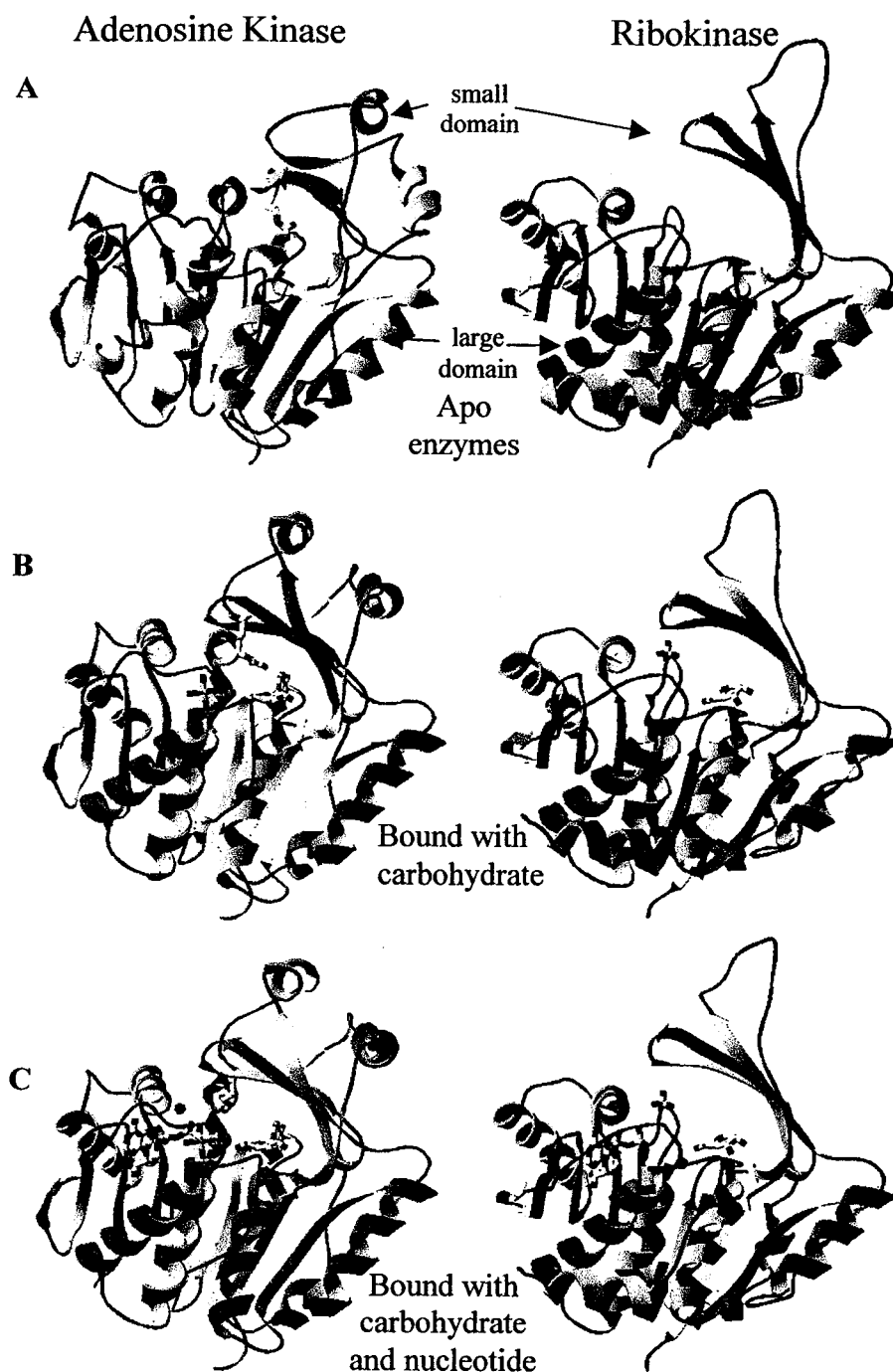
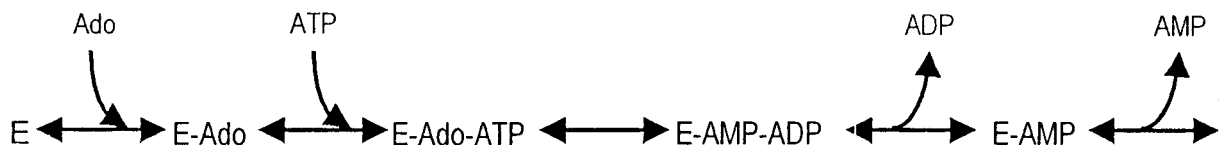


Figure 1.3 Structures of AK from *T. gondii* and RK from *E. coli*.

The similarities between *T. gondii* AK (left) and bacterial RK (right) are shown. The small domain acts as a lid over the active site, rotates toward the large domain forming the ATP binding site. A) Apo enzymes; B) bound with adenosine and MO_6 (AK), ribose and phosphate (RK); C) AK bound to adenosine, MO_6 , ACP-PMP and magnesium (pink), and RK bound to ribose, phosphate, ADP and magnesium (pink). Arg 136 of AK translocation into the active site upon nucleotide binding is highlighted. Coordinates obtained from the PDB (Accession numbers for Figs. A, B, and C respectively: 1LIO, 1DGM and 1LII for AK; 1RKA, 1GQT and 1RKD for RK) and modeled with Swiss-PdbViewer.

domain rotation relative to each other [80;82]. Upon substrate binding, adenosine induces a 30° domain rotation for TG AK and ribose induces a 17° domain rotation for RK (Fig 1.3 C and D). The smaller rotation of the RK enzyme is presumably a result of its dimeric form.

Domain closure is stabilized by H-bonding between a highly conserved threonine residue of motif 2 to a small domain main-chain oxygen of either glycine 106 (RK) or lysine 134 (TG AK). This domain rotation creates enhanced interactions between ATP and protein, suggesting an ordered binding of co-substrates [80;82]. Co-substrate 1 (adenosine or ribose) is the first substrate to bind and AMP is the last product to be released, as shown below for AK:



1.1.2.4 Motif 2

The second motif functions as a novel P-loop which points away from the catalytic site for both apo-enzyme forms of AK and RK. Small movements within the residues of motif 2 for RK is associated with lid movement, where the closing of the lid induces this P-loop to point towards the active site, possibly increasing enzyme affinity for ATP (Fig 1.3). Adenosine binding to TG AK induces a coil to helix transition of 4 residues preceding the P-loop, which in turn cause the next 4 main-chain nitrogens to undergo a helix to coil transition which create an anion hole [80;82]. The structure of the human enzyme lacks an anion hole structure, suggesting that a β - or γ -phosphate group

is required for formation. The binding of AMP-PCP to TG AK (a non-hydrolyzing analogue of ATP) induces the small domain residue arginine 136 to rotate into the active site and form H-bonds with two non-bridging γ -phosphate oxygens, further stabilizing the anion hole [80]. This anion hole is thought to be important for transition state development of phosphate transfer. Within this P-loop is an absolutely conserved familial aspartic acid residue. This residue is found within 2.8Å of the 5' hydroxyl of the substrate undergoing phosphorylation (adenosine for AK and ribose for RK), therefore suggested to function as the catalytic base [37;80-82].

1.1.2.5 Ligand Binding

The structures of AK and RK depict that adenosine and ribose occupy similar 3D space at their respective active sites. Both carbohydrate ribosyl ring oxygen O4' atoms adopt an unusual *endo*-puckering which is seen only 7% in solution. More commonly, C2' or C3'-*endo* puckering is seen in solution, which are energetically more favorable (Fig 1.4). The original human AK structure was crystallized with two molecules of adenosine, the second assumed to represent ATP binding based on similarity to ADP bound by RK. This assumption was confirmed with the structure of TG AK, which shows AMP-PCP bound at the adenosine 2 site.

The tertiary structures of AK and RK have also revealed the binding of magnesium and inorganic phosphate. The structure of bacterial RK was resolved with ribose, ADP, and a bound phosphate near the active site [81]. The distance of the phosphate ion from the β -phosphate of ADP suggests that this phosphate does not

represent the γ -phosphate binding site. This phosphate ion makes close contacts with conserved asparagine and glutamic acid residues at sequence positions 187 and 190 respectively. These conserved residues are also found in human short AK in similar 3D space, residues 223 and 226. Sequence analysis of proteins belonging to the PfkB family of carbohydrate kinases shows that the sequence motif NXXE is highly conserved (Fig. 1.2).

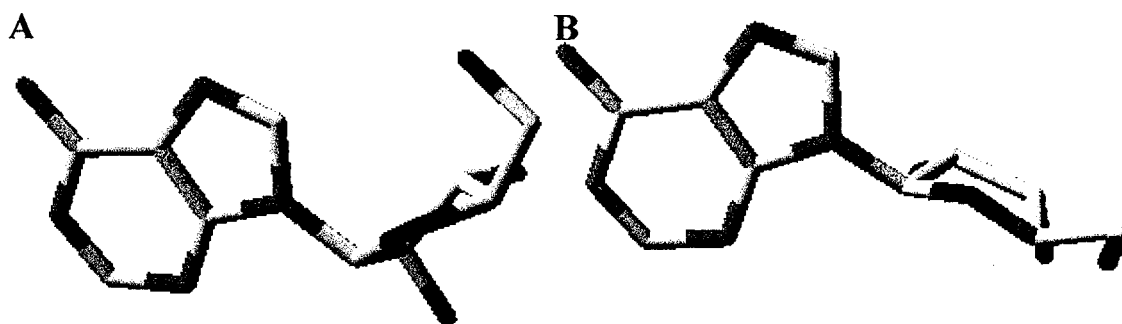


Figure 1.4 Unusual O4'-endo Puckering of Adenosine.

The 3D structures of: A) adenosine which adopts the common C3'-*endo* conformation and; B) adenosine as seen in the active site of adenosine kinase, which adopts an unusual O4'-*endo* conformation. The unusual puckering of the ribose ring is as that which is seen for ribose in the structures of ribokinase.

The human short AK protein was crystallized in the presence of adenosine and magnesium. The structure shows a magnesium ion, which has typical octahedral coordination to six ordered waters (MO_6), bound at the active site [37]. Five of these six ordered waters make H-bond interactions with the protein, which include side chain contacts with conserved NXXE residues asparagine 223 and glutamic acid 226 as well as with the putative catalytic base, aspartic acid 300. The position of MO_6 differs from the magnesium ion found in the active site of TG AK, which is coordinated between the α and β phosphates of AMP-PCP [80]. The position of the TG AK magnesium ion was assigned based on its stable behavior during thermal parameter refinement, though the electron density does not show octahedral coordination, which is characteristic for magnesium.

1.1.3 Isolation and Characterization of Adenosine Kinase Mutants

Adenosine analogues, such as toyocamycin and tubercidin, are toxic to mammalian cells and inhibit the growth of bacteria, fungi, parasitic organisms, and viruses. Cells which are resistant to toxic levels of adenosine and analogues are found to be deficient in AK [55]. Thus, the cytotoxicity of these compounds is correlated with phosphorylation through AK [34]. Analogues such as toyocamycin and tubercidin, similar to adenosine, contain a carbon-nitrogen bond in the ribosidic linkage (N-nucleosides, Fig. 1.5 A). Another group of analogues such as formycin A and B, contain a carbon-carbon bond in the ribosidic linkage (C-nucleosides, Fig. 1.5 B). In 1978, it was reported that Chinese hamster ovary (CHO) cells which were stably resistant to N-nucleosides were obtained spontaneously at unusually high frequencies, 100 – 10,000

times higher than that which is typically observed, both in the presence and absence of mutagenic agents. Similar resistance to toyocamycin was seen in a number of CHO cell lines [83]. The unusually high frequency of CHO cell resistance to N-nucleosides was confirmed by others in 1979 [84]. All mutants tested were found to be deficient in AK activity, thus the AK locus in CHO cells may correspond to 'a mutational hot spot' [83]. Mutants resistant to N-nucleosides also show a high degree of cross resistance to C-nucleosides [85]. This group of mutants is referred to as Class A. Southern and northern blot analysis suggest gross structural alterations of the AK gene in Class A mutants [25].

Further studies indicated that a distinct group of mutants could be isolated by selecting CHO cells in the presence of C-nucleosides formycin A and B [85]. This novel group of mutants showed a degree of cross resistance with other C-nucleosides but not with N-nucleosides. This group of mutants is referred to as Class B. The frequency of Class B mutants was not abnormally high as seen with the Class A mutants [86]. Adenosine, which is also an N-nucleoside, is readily taken up by this group of mutant cells, though AK activity could not be detected with cell extracts [85;86]. Gross structural alterations of the AK gene for Class B mutants are not observed [25]. Hybrids formed between formycin resistant and formycin sensitive cells retained resistance to C-nucleosides, although they contained approximately 50% AK activity [86].

The gene structure for human and Chinese hamster (CH) AK as well as the nature of genetic lesions for Class A mutants has recently been solved [87]. The mammalian AK gene is extremely large, the human gene is estimated to be >500 Kb and

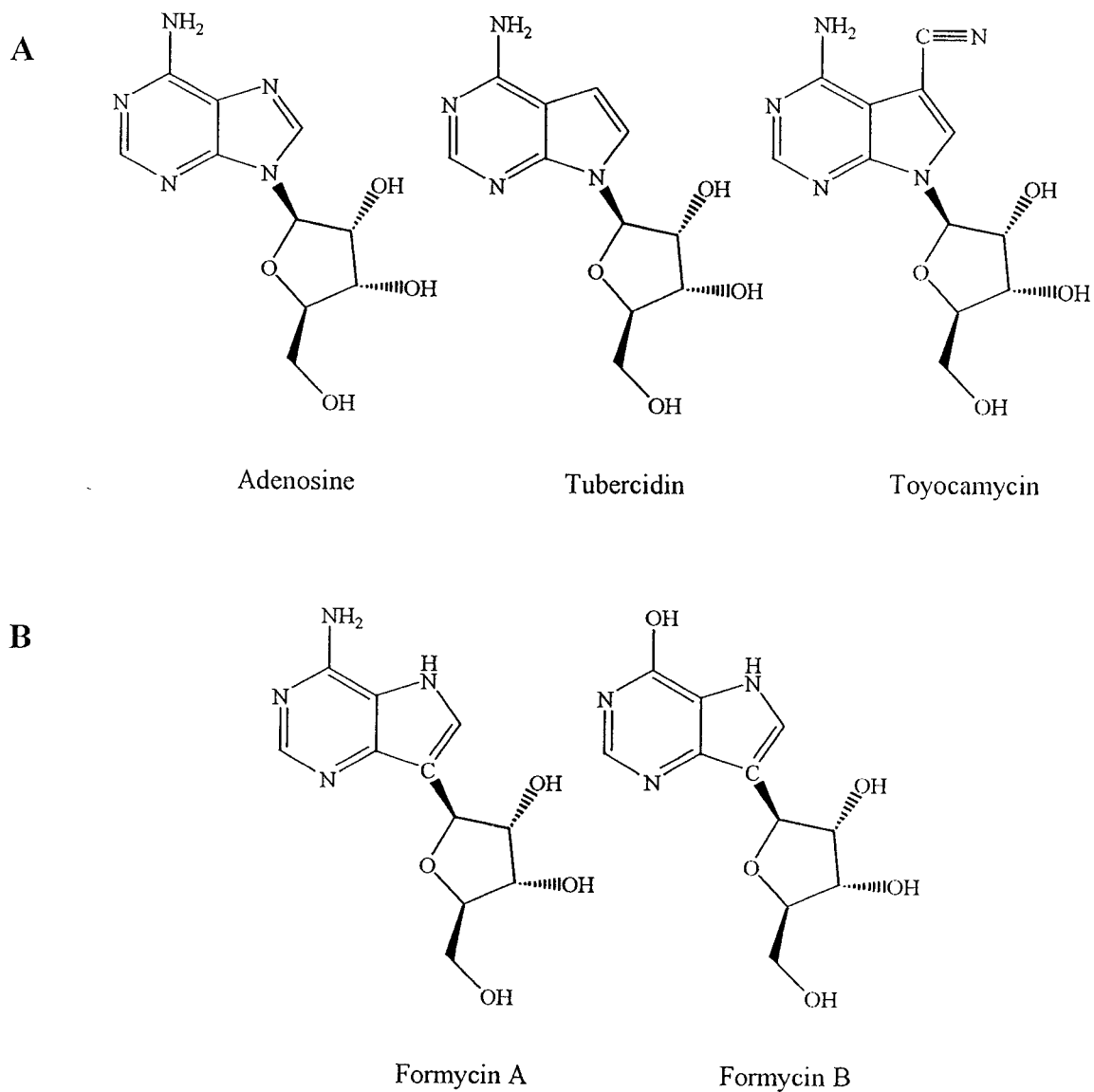


Figure 1.5 Examples of N- and C-nucleosides.

The structures of adenosine analogues are compared to the structure of adenosine. A) Examples of N-nucleosides and B) Examples of C-nucleosides. The structures were created with CS ChemDraw Pro version 4.5.

CH AK estimated to be >150 Kb. Though exact length of the 10 introns have not been determined, most are indicated to be greater than 15 Kb. Of the 11 exons for human and CH AK, their length ranges from 36 to 765 bp. The coding sequence for CH AK is about 1.1 Kb. The transcripts for two Class A mutants, one selected with tubercidin and the other with toyocamycin, were much smaller, 0.6 and 0.4 Kb respectively. The tubercidin mutant shows the deletion of exons 5 through 8. The toyocamycin mutant has lost exons 2 through 8.

During the determination of the AK gene structure, it was observed that there is a close linkage between the CH AK gene and the gene for the clathrin adaptor mu3 protein (beta 3A subunit of the heterotetrameric AP-3 protein complex). The gene for clathrin adaptor mu3 is encoded on the opposite strand and linked to AK in a head to head fashion, separated by <200 base pairs. It is thought that both AK and clathrin adaptor mu3 proteins share a common promoter [87].

1.2 Adenosine

The term 'purine' was coined by chemist and Nobel laureate Hermann Emil Fischer in the late 19th century, from the Latin 'puris' (pure, clean) and 'uricus' (uric acid). The purine adenosine is an autacoid, or 'local hormone', which modulates a broad range of physiological responses in mammalian tissues via interaction with adenosine receptors. As the half-life of adenosine is of the order of seconds, its actions are localized to the site of cellular release. After more than 6 decades of research, the complexities of adenosine production and metabolism are now becoming clear. Enzymes which catalyze

the production and metabolism of adenosine are found in both the cytosol and extracellular regions. Under normal oxygenated conditions, the rate of ATP catabolism and metabolism are equal. The stable concentration of ATP is 5-10 mM and ADP is 40-60 μM [43]. This high cytosolic ATP/ADP implies a low cytosolic AMP concentration. An increase in the concentration of AMP indicates a threat to the ATP supply. When the oxygen supply is inadequate for oxidative phosphorylation of ADP to regenerate ATP, such as during ischemia or hypoxia, the ATP/ADP ratio is compromised. The increasing concentrations of ADP induce adenylate kinase activity in the direction of AMP formation to keep ADP/AMP ratio stable. Thus, there is a net increase in the concentration of AMP. AMP is then hydrolyzed to adenosine [88;89]. The cytosolic concentration of adenosine is kept in the nanomolar range by AK and AD. The physiological concentration of adenosine in mammalian tissues is 0.1-1 μM [90;91] with a half-life of 0.6-1.5 seconds in interstitial fluids [92].

Under conditions of tissue stress or trauma, such as ischemia or hypoxia, the local intracellular production of adenosine is dramatically increased [93-96]. Increased cytosolic concentrations results in the net cellular release of adenosine into interstitial fluids, passing to extracellular space *via* a bi-directional facilitated diffusion transporter [97;98]. Interstitial concentrations of adenosine have been found as high as 30 μM within the myocardium [99], 30 μM in the brain [100], and 100 μM in the spinal cord [101] during an hypoxic episode.

When there is an excess breakdown of ATP over formation, adenosine functions as a 'retaliatory metabolite' to restore energy balance by a variety of actions

upon binding to adenosine receptors [102]. The protective effects of adenosine include coronary vasodilation, reduction of neuronal activity, inhibition of platelet aggregation, anti-inflammatory activity, angiogenesis and ischemic preconditioning [103-109]. Inhibition of AK has been shown to increase local concentrations of adenosine and enhance this protection [43;110-116].

1.2.1 Adenosine Metabolism

Recent studies have been performed in order to enhance the protective effects of endogenously accelerated adenosine release. The focus is to inhibit adenosine metabolizing enzymes, thereby circumventing non-specific side effects resulting from P1 receptor agonist administration. Inhibition of both adenosine kinase and adenosine deaminase has been shown to facilitate the endogenous increase in extracellular levels of adenosine under conditions of oxygen deprivation.

The free cytosolic concentration of adenosine under normoxic conditions had been estimated to range between 0.01-0.1 μM [117;118]. The concentration of adenosine is tightly regulated by AK and AD, which catalyze the phosphorylation and deamination of adenosine respectively. Adenosine deaminase has at least three isoforms which have been found in plasma [119], cytosol and bound to membrane on the cell surface [120]. Adenosine deaminase has a *K_m* for adenosine ranging from 25-150 μM [121-124].

AK activity is localized to the cytosolic region [123], has a *K_m* for adenosine of approximately 1 μM and becomes inhibited at concentrations above 10 μM at physiological pH [53;65;123]. However, it has been reported that AK experiences a

biphasic pH optimum at concentrations of adenosine greater than 25 μM [18]. *In-vivo* experiments with AK and AD indicate that a depression in pH (mimicking ischemic acidosis), from pH 9.0 to 5.5, reduces the catalytic efficiency of AK, yet it consistently remains two-fold higher than that of AD [124]. Thus, the primary enzyme which regulates adenosine under physiological and ischemic conditions is adenosine kinase [11;124].

1.2.2 Adenosine Production

Quantitatively, the most important source of adenosine under physiological conditions is the recently characterized AMP-selective cytosolic 5'-nucleotidase which is designated as cN-I [125;126]. The increase in adenosine under conditions of ischemia occurs by hydrolysis of AMP via both cytosolic and membrane bound ecto-5'-nucleotidase [127]. Hydrolysis of S-adenosylhomocysteine by SAH hydrolase further contributes to the total adenosine pool (Fig. 1.6) [128].

1.2.2.1 Cytosolic-5'-Nucleotidase-1

Historically it was thought that the production of adenosine was catalyzed by the activity of cytosolic-5'-IMP-nucleotidase. Only recently has a cytosolic AMP-specific nucleotidase (cN-1) been identified, cloned [125], and characterized through the use of selective inhibitors [126]. These recent developments have led to the determination that cN-1 is responsible for approximately 76% of adenosine production in mammalian cells. *In vitro* studies have shown that cN-1 is activated by ADP but inhibited by ATP

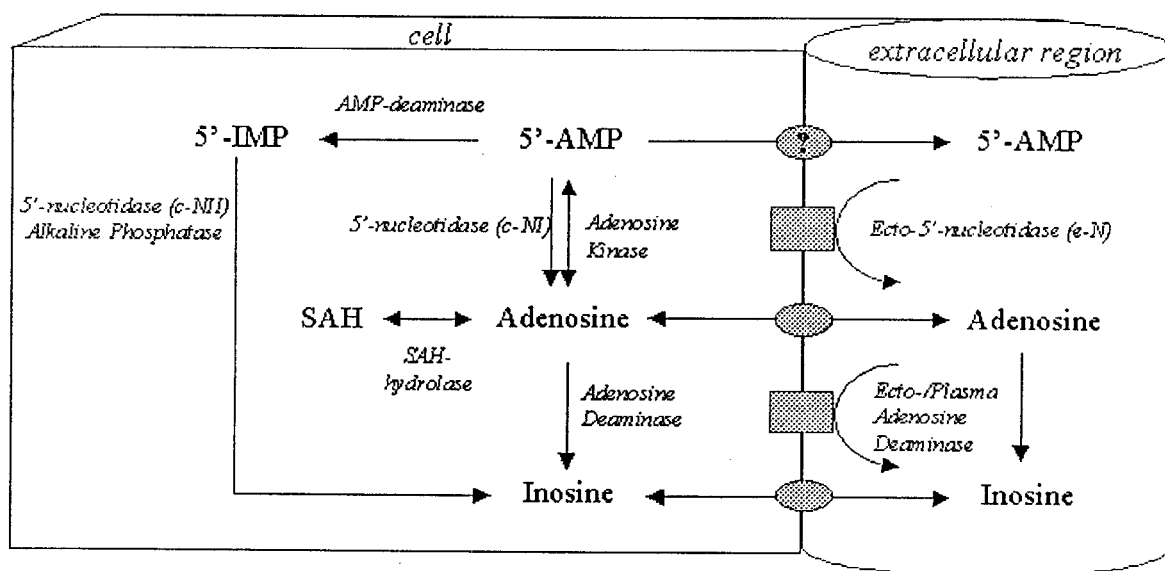


Figure 1.6 Adenosine Production in the Cell

The pathways and enzymes of production and metabolism of adenosine currently know. This figure is adapted from Deussen (2000) [129]. Enzymes are italicized. Shaded boxes represent membrane bound proteins and shaded ellipses represent bi-directional facilitated diffusion transporters.

suggesting that it is regulated by ATP catabolism [88;130]. cN-1 has also been found to require Mg^{2+} for activity and is inhibited by both inorganic phosphate and acidic pH [130-134]. More recently, pH has been identified as the major regulator of cN-1 activity in an isolated heart during ischemic acidosis [88]. Therefore, during the onset of ischemia, it appears that the increasing concentration of ADP stimulates the activity of cN-1, whereas later under conditions of sustained ischemic acidosis the activity of cN-1 is decreased. Increases in cellular phosphate concentrations upon prolonged ischemia further inhibit activity. This suggests a role for cN-1 in mediating ATP degradation and facilitating the re-synthesis of ATP from AMP pools upon reperfusion [88]. However, additional quantitative analysis which includes net adenosine production and inhibition of all sources of adenosine metabolism are required to verify this hypothesis.

1.2.2.2 *Ecto-5'-nucleotidase*

A secondary source of adenosine occurs *via* hydrolysis of AMP to adenosine by the enzyme ecto-5'-nucleotidase (e-N) [135]. e-N is a glycoprotein which is found primarily in the plasma membrane of eukaryotic cells [136-138] with the catalytic site exposed to the extracellular region [139;140]. Cells which are well equipped with e-N are heart cells, including myocytes, endothelial and fibroblast cells [141]. The contribution of e-N to the total production of adenosine in mammalian cells is estimated to be between 24-30% [27;142]. A number of *in vitro* studies have shown that e-N is inhibited by both ATP and ADP [140;143;144]. The production of adenosine via the extracellular glycoprotein has been shown to become enhanced under hypoxic conditions [127;145]. These data, along with reports that the activity of cytosolic cN-1 is reduced during ischemic

acidosis, suggest that the activity of e-N becomes increasingly important under conditions of prolonged ischemia. Equal contribution of endo and ecto-5' nucleotidase has been shown under conditions of ischemia [146].

1.2.2.3 SAH

In addition to hydrolysis of 5'-AMP, adenosine can also be produced from S-adenosylhomocysteine (SAH) *via* SAH-hydrolase (EC 3.3.1.1). SAH is derived from S-adenosylmethionine (SAME), by the donation of a methyl group, in reactions catalyzed by a large number of methyltransferases. Low levels of adenosine and homocysteine are constantly produced through the SAH pathway independently of tissue oxygenation [147]. The reaction catalyzed by SAH-hydrolase is reversible and the direction of activity is dependant on the local concentrations of homocysteine [148]. The enzyme favors the hydrolysis of SAH as both adenosine and homocysteine are rapidly metabolized [149]. The contribution of this pathway to the adenosine pool under conditions of ischemia is quantitatively negligible [147;150]. Homocysteine is metabolized *via* transmethylation or transsulfuration [151]. Transmethylation is catalyzed by either methionine synthase or betaine homocysteine methyl transferase. Transsulfuration is catalyzed by cystathionine β -synthase leading to cystathionine. Elevated levels of homocysteine have been linked to cardiovascular [152] and neural diseases [153].

1.3 Purine Salvage

De novo purine synthesis begins with the conversion of ribose-5-phosphate to 5-phospho-D-ribosyl-1-pyrophosphate (PRPP) by PRPP synthetase and ATP. The conversion of PRPP to AMP requires some 13 enzyme catalyzed reactions, 4 molecules of ATP and 1 molecule of GTP (Fig. 1.7). The salvage of adenosine nucleotides from pre-formed bases is energetically more favorable than *de novo* synthesis. As previously mentioned, adenosine production can occur through a number of independent pathways. AMP can then be salvaged from adenosine *via* AK. AMP can also be salvaged through the adenine phosphoribosyltransferase (APRT) pathway (Fig. 1.8), beginning with SAMe.

APRT, like adenosine kinase, is also found in purine auxotrophs, which include parasitic organisms. Both AK and APRT have been shown to undergo conformational changes upon the binding of ligands [80;154]. The recycling of AMP through purine salvage pathways is thought to play an important role during periods of rapid cell division as with tumor proliferation [155]

1.4 Adenosine Receptors

In 1929, Drury and Szent-Gyorgyi first discovered that adenosine influenced several physiological activities including increased coronary blood flow, reduction of heart rate and blood pressure and induction of sleep [156]. Decades later, investigators realized that the effects of adenosine could be mimicked with adenosine analogues suggesting the presence of adenosine receptors in heart [157] and brain [158].

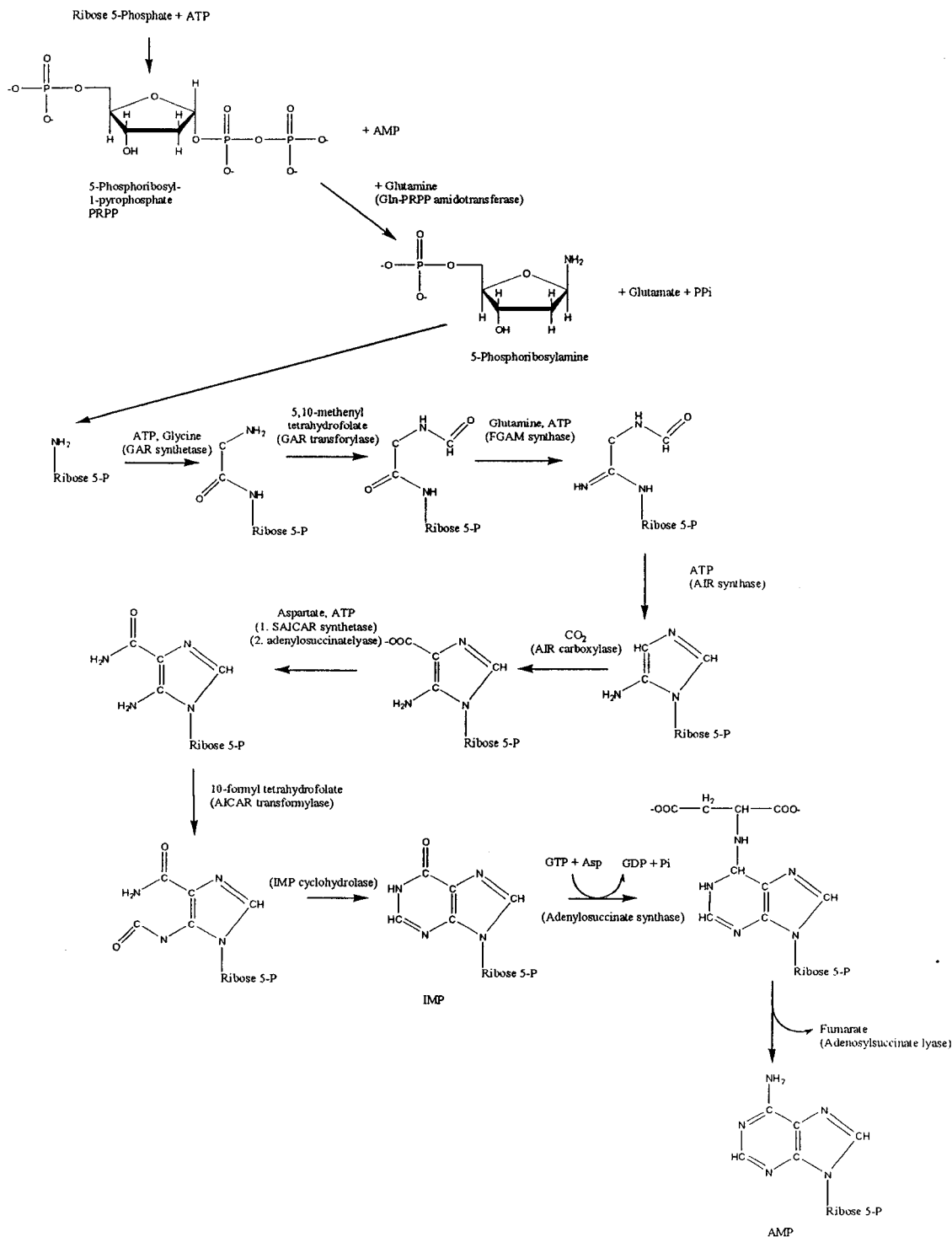


Figure 1.7 De Novo Pathway of AMP Synthesis

De novo purine synthesis from PPRP requires 13 enzyme catalyzed reaction. The structures of the intermediates are shown. The enzymes which catalyze the reactions are shown in brackets.

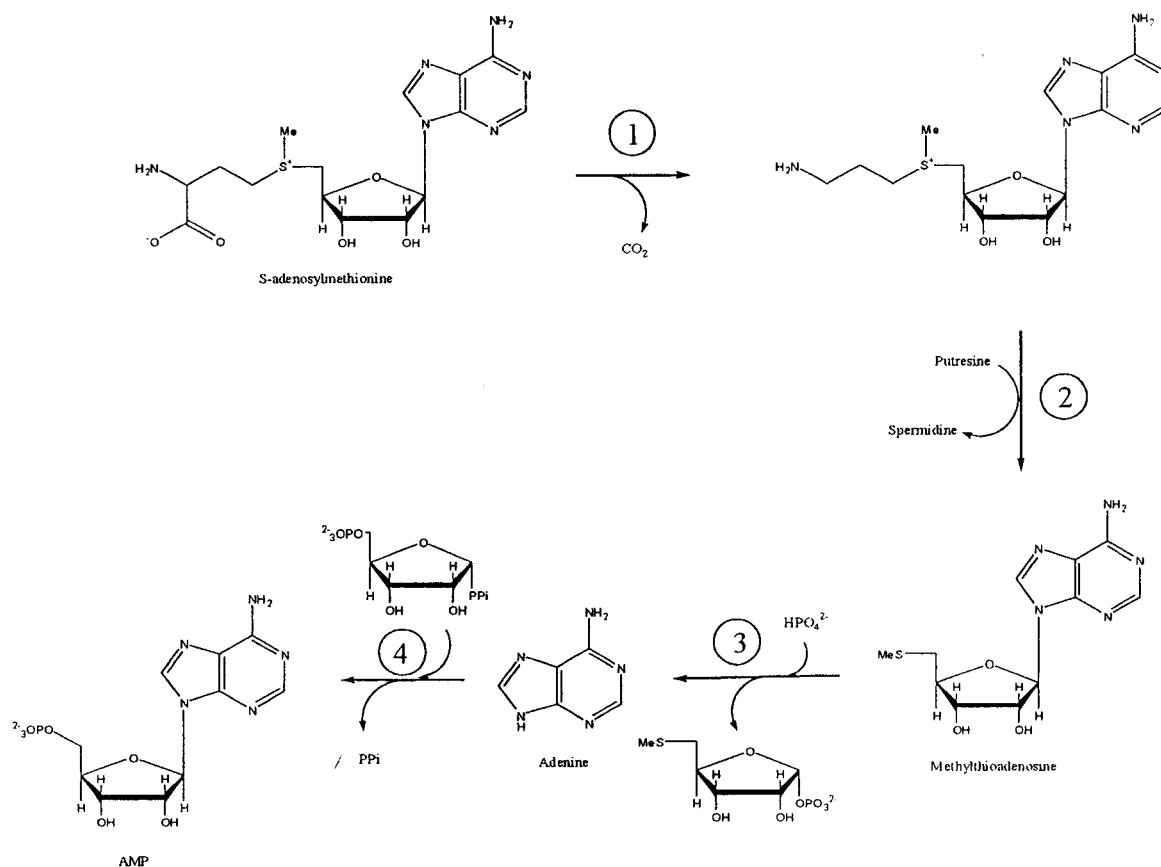


Figure 1.8 Purine Salvage from S-adenosylmethionine to AMP.

The pathway of purine salvage from S-adenosylmethionine thru to AMP begins with the reaction of: 1) S-adenosyl decarboxylase; 2) spermidine synthase (one example of polyamine synthesis); 3) methylthioadenosine phosphorylase; and 4) adenine phosphoribosyltransferase (APRT).

Figure adapted from Shi *et al.* 2001 [154].

In the late 1970's through to the next decade, two major types of adenosine receptors were identified, classified as A1 and A2. Through G-protein coupling, A1 was found to inhibit, and A2 to stimulate, the activity of adenylate cyclase [159-161].

Molecular biology allowed for the cloning and characterization of a number of G-protein coupled receptors in 1989 [162], two of which were later identified as adenosine receptors. Agonists developed to evoke unique responses in specific cells or tissues in combination with radio-ligand binding have currently identified four adenosine, or purinergic receptors (P1). These adenosine-specific receptors, A1, A2a, A2b and A3 are now cloned [163-166]. Most cells express all four receptor subtypes, however their population is dependant on cell type.

It is now well documented that adenosine elicits cardio and neuro protection through P1 receptor interaction, in cell cultures, brain slices, and animal models. Adenosine release during cardiac ischemia provides receptor mediated vasodilatory protection and is shown to mediate ischemic preconditioning (a brief period of ischemia resulting in increased tissue viability during subsequent prolonged ischemia) [93;167]. Infarct size, or area of tissue death due to a lack of oxygen, is reduced by adenosine both in the heart [168] and brain [169] [111]. Increased concentrations of adenosine has also been correlated with a decrease in the rates of ATP catabolism and intracellular H^+ and Ca^{2+} accumulation during ischemia [170;171].

An overwhelming number of studies have been performed to determine the effects of each P1 receptor through the use of agonists and antagonists. While a great number of studies have been performed to elucidate the roles of individual P1 receptors

in the heart, the most complete data is found for P1 receptors in the brain. At the time of this document, with the exception of a specific antagonist for the A2B receptor, a complete set of receptor-specific agonists and antagonists exist. Because of the broad range effects of P1 receptor activation, a number of review articles on the subject [172-174] freely admit that it is impossible to completely summarize all recent developments, even when limited to a specific tissue type within one year. A brief overview of the principle roles of each receptor subtype follows.

1.4.1 A1 Receptors

For centuries, people have known that drinking large amounts of coffee will cause an accelerated heart rate and keep you awake. This is due to caffeine antagonism of A1 receptors in the heart and brain. Many selective agonists and antagonists to A1 receptors have been developed over the past few decades. A1 receptor activation is consistently shown to have a protective role under conditions of oxygen deprivation and play a major role in ischemic preconditioning [32;175-177]. A1 receptors, characterized from brain tissue have the highest affinity for adenosine at roughly 70 nM [178].

1.4.2 A2A Receptors

The major role of A2A receptor activation is in the regulation of blood flow. A2A receptors have been found to mediate vasodilatation, leading to the increased delivery of substrates and oxygen to under-perfused tissues [179;180]. The expression of A2A receptors is induced by hypoxia [181], therefore activation of these receptors may be important in cases of prolonged hypoxia. Activation of A2A receptors produce a

number of anti-inflammatory responses which protects the heart and brain by limiting reperfusion injury [182-184]. Other roles for the A2A receptor in tissue protection remain controversial as both agonists and antagonists have been shown to elicit protective effects. The high-affinity A2A receptors and low-affinity A2B receptors, characterized from brain tissue, have an affinity for adenosine in the range of 150 nM and 5100 nM respectively [178].

1.4.3 A2B Receptors

Activation of A2B receptors has been shown to attenuate neutrophil induced endothelial cell damage by diminishing neutrophil adhesion to vascular endothelial cells and stimulates nitric oxide release from vascular endothelial and smooth muscle cells [185;186]. A2B receptors expression is induced by IFN-gamma, and stimulation of A2B receptors have been shown to inhibit IFN-gamma induced expression of nitric oxide synthase suggesting A2B and IFN-gamma involvement of a feedback mechanism for macrophage deactivation [187].

1.4.4 A3 Receptors

The A3 receptor is the most recently identified sub-type. It has been shown that agonists to the A3 receptor show neuro-protection only when administered during the late stages of ischemia, when endogenous adenosine concentrations are high enough for receptor activation [188]. Activation of this receptor has also been shown to have long-lasting protection from ischemic injury in the ventricle heart cell up to 45 minutes after

exposure to agonist [189]. The A3 receptor of the brain has the lowest affinity for adenosine at approximately 6500 nM [178].

1.4.5 Therapeutic Utilization of Adenosine Receptor Activation

The therapeutic utilization of adenosine receptor activation is still quite new, however adenosine itself is currently being used to regulate the heart in the treatment of supraventricular tachycardia (accelerated heart rhythm) under the trade name Adenocard®. Adenosine is also used as a tool to diagnose cardiac abnormalities under the trade name Adenoscan® by producing vasodilation of coronary arteries. However, the systemic administration of adenosine or specific receptor agonists have been found to give rise to adverse reactions such as facial flushing, lightheadedness, paresthesia (abnormal neurological sensations such as numbness, tingling, burning, prickling and increased sensitivity), headache, diaphoresis (profuse perspiration), palpitations, chest pain, hypotension, nausea, metallic taste, and shortness of breath. Chronic administration of certain receptor agonists has been shown to slightly increase cell death in animal models of global forebrain ischemia [190].

1.5 Inhibitors of Adenosine Kinase as Therapeutic Agents

Adenosine has a short half-life in biological fluids and its beneficial effects are limited to tissue sites undergoing cellular release. It is hypothesized that the inhibition of adenosine metabolizing enzymes will increase the endogenous concentration of adenosine. As AK is the principle enzyme responsible for adenosine metabolism, it is thought to be a promising candidate for the development of therapeutic agents. The

inhibition of AK has been found to effectively increase the concentrations of intra and extra-cellular adenosine, enhancing its clinical benefits in the absence of systemic side effects seen with direct acting receptor agonists.

Analogues of 5-iodotubercidin (5IT), such as GP683 (Fig. 1.9), are potent inhibitors of AK and have been shown to have more effective anti-seizure activity in animal models of maximal electric shock induced seizures [191;192] than inhibitors of either adenosine deaminase or adenosine transport [193]. Intervention with 5-deoxy- 5IT prior to and immediately after middle cerebral artery occlusion (MCAO) in rats resulted in significant reduction of infarct size [111]. Administration of GP683, delayed until 90 minutes after MCAO in rats, has also resulted in a substantial reduction of infarct size [194]. 5IT analogue A-134974 (Fig. 1.9) has been shown to effectively reduce nociception in animal models of pain [195;196]. A novel, potent, non-nucleoside AK inhibitor ABT-702 (Fig. 1.9) has recently been found to have analgesic and anti-inflammatory properties in mouse models of pain and arthritis [197;198]. This compound has been found to be orally active, and is a competitive inhibitor with respect to adenosine *in vitro*, with $IC_{50}=1.7$ nM *in vivo* [199].

Inhibitors which can be metabolized by AK have limited use *in vivo* because of their short half-lives in interstitial fluids. Also, their therapeutic utility is hampered by the fact that they can potentially form cytotoxic metabolites. Non-nucleoside inhibitors of AK which are not metabolized to cytotoxic compounds are promising agents to increase the endogenous concentration of adenosine. A greater understanding of enzyme mechanism is clearly required. Ligand binding studies of AK from a variety of

mammalian sources will further support the design of non-nucleoside inhibitors which do not form cytotoxic compounds.

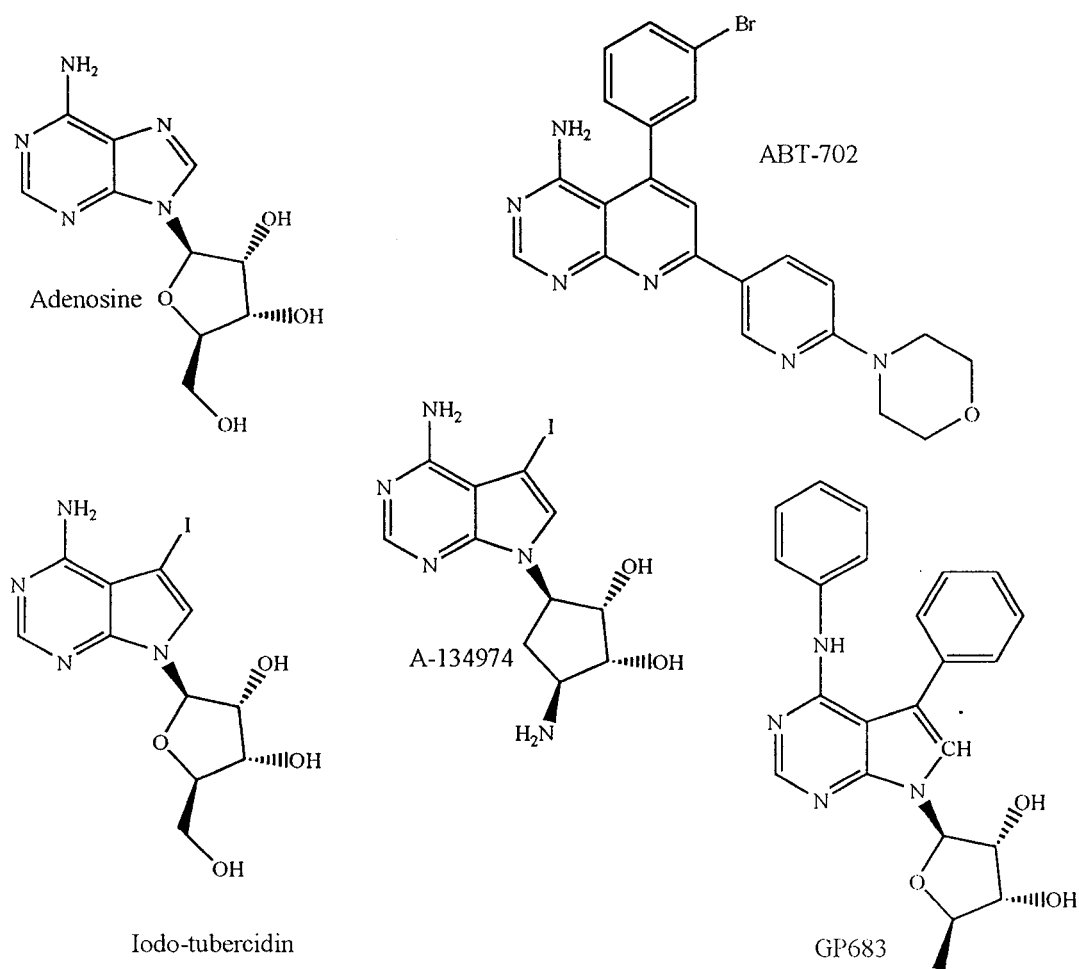


Figure 1.9 The Structure of AK Inhibitors.

The structure of AK inhibitors with therapeutic potential in animal models are shown and compared to the structure of adenosine

1.6 Purpose of this Study

Complete understanding of the mechanism of AK activity is a lofty goal, when one considers the conflicting data reported by a large number of proficient investigators. Not only has the conformation of the enzyme been shown to change upon the binding of each substrate, biochemical investigation is hampered by the fact that AK is inhibited by high concentrations of all substrates. The recent observation that pentavalent ions could stimulate the activity of mammalian AK and reduce the K_m for adenosine, not ATP, and is not consumed in the reaction, has further contributed to un-answered questions regarding the mechanism of activity and order of ligand binding.

The primary goal of this study is to provide insights as to the role of pentavalent ions to the activity of AK. Understanding the influence of pentavalent ions is interesting for a number of reasons. 1) Stimulation of AK activity by pentavalent ions had not previously been reported though inorganic phosphate is a contaminating agent in commercial preparations of ATP. 2) The influence of pentavalent ions may be a conserved mechanism for AK from all sources, as well as other members of the PfkB family of carbohydrate kinases. 3) Pentavalent ions may bind near the active site which may be an important target for the development of selective inhibitors.

A secondary goal of this study was to determine the role of different structural regions of the protein. 1) The greatest divergence in the primary sequence among AK from a number of sources, and within human isoforms, lay in the extreme N-terminus (within exon 1 of human and CH AK). 2) Conserved motif 1 is located near the N-

terminus and conserved motif 2 lay near the C-terminus. 3) Adenosine 1 of the human AK structure interacts with residues near the N-terminus and adenosine 2 interacts with residues near the C-terminus providing little information as to which site represented the ATP binding site. 4) An aspartic acid residue found in motif 2 is absolutely conserved among all members of the PfkB family and has been hypothesized to be instrumental for catalytic activity.

The third goal of this study is to compare the activities of AK from a variety of sources under similar conditions for a variety of reasons. 1) It is most likely that differing reports of enzyme activity and substrate binding properties are a result of data generated at various sub-saturating substrate concentrations and different buffers. 2) Substrate binding affinities for AK from a variety of sources can be compared and may eventually be related to differences in their structure once 3D structures of AK from a variety of sources are revealed.

The initial strategy was to perform detailed analysis of the effect of phosphate on the kinetic parameters with AK from cellular extracts and purified from mammalian tissue, and compare this to the activity of recombinant protein. This would set a basis for further studies of: 1) deletion studies of the recombinant protein; 2) Site-directed mutagenesis studies of the recombinant protein; 3) Comparison of activity and pentavalent ion effects for AK from a variety of sources; and 4) Comparison of the effects of pentavalent ions on recombinant AK and recombinant ribokinase.

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Chapter 2. The Influence Of Inorganic Phosphate On The Activity Of Adenosine Kinase

Mary C. Maj, Bhag Singh and Radhey S. Gupta (2000) *Biochimica et Biophysica Acta*, 1476, 33-42.

2.1 Preface

Pentavalent ions had previously been shown to stimulate activity of mammalian adenosine kinase and decrease the K_m for adenosine. In order to better understand the effect of pentavalent ions on adenosine kinase activity, a more detailed analysis was performed. These studies were performed at a number of phosphate concentrations at physiological pH and at pH 6.2. The study of adenosine kinase is physiologically relevant at both these pH values, the latter of which mimics conditions of ischemia. Under tissue stress and trauma such as myocardial or cerebral ischemia, there is an extreme increase in the local concentration of adenosine which is correlated with depression in pH to as low as 5.5. Little is known about the activity of adenosine kinase under these conditions.

The results of this work shows that inorganic phosphate not only increases the activity and decreases K_m for adenosine in a dose-dependant manner, but it also increases the K_i for adenosine at both pH values studied. At more acidic pH, the activity of AK is greatly reduced, with a 10 fold increase in K_m .

All experimental work outlined in this publication was performed by myself except for the construction of the expression vector containing the cDNA of CHO AK. The tables contained in this publication have been altered to include the standard deviation of calculated kinetic parameters. The first and second order rate constants, k_{cat} and k_{cat}/K_m , have also been included. The figures presented in this paper now contain

the standard error of initial velocity. Fluorescent emission spectrum of CHO AK, which was not shown in the paper, is also included as Figure 2.9.

Following the publication of this paper, 3D structures of *Toxoplasma gondii* AK was solved with various ligands, revealing a number of conformational changes that occur upon the binding of each co-substrate. Chapter 5 shows evidence that the activity of adenosine kinase is influenced by high concentrations of both co-substrates, magnesium, pH and pentavalent ions. Given the complexity of steady-state parameters, K_m is likely a kinetic rather than a binding constant.

2.2 Paper

The influence of inorganic phosphate on the activity of adenosine kinase

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2.3 Abbreviations

Ado, adenosine; AK, adenosine kinase; BL, beef liver; CHO, Chinese hamster ovary; Pi, inorganic phosphate

2.4 Abstract

The enzyme adenosine kinase (AK: EC 2.7.1.20) shows a dependence upon inorganic phosphate (Pi) for activity. The degree of dependence varies among enzyme sources and the pH at which the activity is measured. At physiological pH, recombinant AK from Chinese hamster ovary cells (CHO) and AK purified from beef liver (BL) shows higher affinities for the substrate adenosine (Ado), larger maximum velocities and lower sensitivities to substrate inhibition in the presence of Pi. At pH 6.2, both BL and CHO AK exhibit almost complete dependence on the presence of Pi for activity. The data show that both enzymes exhibit increasing relief from substrate inhibition upon increasing Pi and the inhibition of BL AK is almost completely alleviated by the addition of 50 mM Pi. The affinity of CHO AK for Ado increases asymptotically from K_m 9.5 μ M to a limit of 0.7 μ M upon the addition of increasing Pi from 1 to 50 mM. The concentration of Ado necessary to invoke substrate inhibition also increases asymptotically from K_i 18.6 μ M to a limit of 69 μ M at saturating concentrations of phosphate. In the presence of increasing amounts of Pi, the maximal velocity of activity increases hyperbolically. The effect that phosphate exerts on AK may be either to protect the enzyme from inactivation at high adenosine and H⁺ concentrations or to stabilize substrate binding at the active site.

2.5 Introduction

Adenosine kinase (ATP:adenosine 5'-phosphotransferase, AK; EC 2.7.1.20) is one of the most abundant mammalian purine nucleoside kinases known. This single polypeptide purine salvage enzyme catalyzes the phosphorylation of adenosine to AMP according to the reaction:



The purine salvage pathway involves the synthesis of purine nucleotides from pre-formed purine bases, the product of nucleotide degradation. AK is the first enzyme in the catabolic utilization of adenosine and is instrumental in the regulation of intracellular and extracellular levels of adenosine [1]. An important regulatory characteristic of AK is its potent inhibition by high concentrations of adenosine. Studies indicate that during myocardial and cerebral ischemia, there are marked increases in adenosine [2;3], inorganic phosphate [4-6] and H^+ concentrations [5;6]. Elevated levels of adenosine are associated with both cardiac [2;7] and neural protection [3;8] against cellular injury resulting from prolonged ischemia. It is thought that agents which enhance the ischemia-induced accumulation of adenosine, such as through the inhibition of AK, could potentially enhance cardio- and neuro-protection [8-10]. However, the activity of AK

under ischemic conditions has not been investigated. This study addresses the regulation of AK activity by inorganic phosphate (Pi) in the presence of inhibitory concentrations of adenosine in an acidic environment.

Several mechanisms for the catalytic activity of AK have been proposed by various groups. Differences in the interpretation of kinetic data are thought to occur due to the regulation of AK by substrate inhibition and inhibition by excess free Mg^{2+} . These properties are interrelated and further affected by pH [11-13]. The pH optimum for activity also varies by altering the Mg^{2+}/ATP^{4-} ratio [14]. It is widely accepted that the mode of AK activity is that of a classic ordered sequential Bi Bi mechanism. However, various investigators have reported differing orders of substrate binding and product release depending on the enzyme source [11;12;15-18]. Structural evidence shows that the adenosine binding site is more buried relative to the putative ATP binding site; it would follow that adenosine binds prior to ATP [19]. Because of their structural similarities between cosubstrates, adenosine may inhibit the enzyme by binding to the Mg/ATP^{2-} site. Therefore, high levels of adenosine may result in competitive inhibition with respect to Mg/ATP^{2-} . Adenosine can be phosphorylated by AK in the absence of ATP by an exchange reaction between adenosine and AMP when ADP is present [17;20]. Thus, at high levels, adenosine may act as an uncompetitive inhibitor by binding to the subsite on the enzyme left vacant after the first product in the ordered reaction sequence has been released. The catalytic and exchange activities of adenosine kinase each show a dependence on Pi or other pentavalent ions such as arsenate and vanadate. The mechanism by which phosphate exerts its effects is not known. It has been shown that Pi

is not exchanged with any of the catalytic reactants, nor is a phosphoryl enzyme intermediate formed [20;21].

Under ischemic conditions, the elevation in adenosine and Pi, as well as acidosis, may all influence the metabolism of adenosine by AK. We have found evidence which suggests that AK becomes increasingly sensitive to substrate inhibition as pH decreases from that of physiological pH. Our studies suggest that Pi increases the affinity of AK for adenosine by relieving the substrate inhibition by adenosine. These studies also indicate that the pH optimum for the catalytic reaction of AK is broadened in the presence of inorganic phosphate.

2.6 Methods and Materials

2.6.1 Materials

[2,8-³H] adenosine (30.1 Ci/mmol) was obtained from DuPont Canada Inc. ATP was purchased from Pharmacia Biotec Inc. Canada. All other chemicals were of analytical reagent grade.

2.6.2 Radioactive Substrate Activity Assay

Adenosine kinase activity was assayed by a stopped radiochemical method using [2,8-³H] adenosine. AK is added to a total volume of 100-600 μ L of reaction mixture. All assays were performed at 35°C. Initial velocities were determined by withdrawing 50 μ L samples at various time intervals. These 50 μ L aliquots are immediately placed in 1 mL of cold LaCl₂ in order to precipitate the radio-labeled AMP product formed in the AK

reaction. The precipitate was collected after a minimum of 3 h by suction filtration on a glass fiber filter and washed with 20 volumes of cold distilled water. The dried glass fiber filters were then placed in 5 ml scintillation vials with aqueous scintillation fluid. The radioactivity is counted on a Beckman LS 7800 scintillator. Unless otherwise stated, assays performed at pH 6.2 contain 20 mM citrate buffer and those performed at pH 7.4 at 35°C contained 20 mM Tris-HCl.

In order to minimize the inhibitory effects of magnesium on the activity of adenosine kinase, it was necessary to hold the concentration of free magnesium at 1 mM for each pH value investigated. Where Mg/ATP is held constant at pH 6.2, the optimum concentration for Mg^{2+} and ATP are 5 mM. These concentrations were calculated to give $[Mg^{2+}]_{free} = 1.04$ mM, $[Mg/ATP^{2-}] = 3.96$, and $[ATP^4] = 1.04$ mM (pK values for ATP are 6.97 and 3.93, dissociation constant for $MgATP^{2-}$ is 0.0143 mM). At pH 7.4 where Mg/ATP^{2-} concentrations remain fixed, the addition of 5.75 mM Mg^{2+} and 5 mM ATP give the calculated values for $[Mg^{2+}]_{free} = 1.04$ mM, $[Mg/ATP^{2-}] = 4.71$ mM and $[ATP^4] = 0.29$ mM. The concentration for adenosine where held fixed is 10 μ M. The pHs of all reagents was standardized to the pH of the assay.

2.6.3 Data Analysis

Radioactivity counts of the radio-labeled AMP formed by the catalytic reaction were plotted against time. The initial velocities, as well as secondary kinetic constants determined from these velocities, are computed with Graphpad Prism graphing software fitted to Eqn. 3.

2.6.4 Protein Purification

Catalytically active recombinant AK from CHO with the addition of a poly-histidine tag was expressed and purified from *Escherichia coli* cells. AK cDNA fragment from CHO cells was obtained as previously described [22] and ligated into a pET 15b vector (Novagen). Verification of insert frame and orientation was obtained by DNA sequencing. These vectors were transformed into *E. coli* strain BL21 (DE3) (Novagen). Overexpression is induced by addition of 0.1 mM IPTG. The bacterial cells were harvested by centrifugation and resuspended in binding buffer consisting of 20 mM sodium phosphate buffer pH 7.8 and 500 mM NaCl then lysed by French press. The lysate was then centrifuged to remove solid debris and then passed through a 0.45 μ m filter. This his-tagged protein was then bound to a nickel resin column (Invitrogen Corp.) which had been blocked with binding buffer containing 500 mM imidazole then pre-equilibrated with binding buffer. The column was washed with three column volumes each of binding buffer at pH 7.8, pH 6.0 and pH 6.3 containing 50 mM imidazole. The protein was eluted with binding buffer pH 6.3 containing 200 mM imidazole. The eluted AK protein was purified to >95% with a specific activity of 1.7 mmoles AMP/min/mg. The final preparation was dialyzed in 20 mM Tris-HCl buffer pH 7.4, lyophilized and stored at -70°C.

The beef liver (BL) enzyme was purified as previously described by Andres and Fox [23] to >95% though the use of anionic and cationic ionic exchange resins, an AMP affinity column and a G75 sizing column. The final preparation was concentrated with a

Centriprep ultrafiltration cell and stored at -70°C in 5 mM glycerol, 1mM DTT and 1mM EDTA. The purified enzyme has a specific activity of 2.2 mmoles AMP/min/mg.

2.6.5 Structural Studies

To test whether Pi evokes a change in the structure of CHO AK, circular dichroism (CD) studies were performed. CD measurements were collected on an Aviv model 60DS spectrophotometer linked to a thermostatted bath. Spectra were collected at protein concentrations of 0.2 or 0.5 mg/ml in 5 mM NaF1 and 5 mM Tris-HCl (pH 7.4) or citrate buffer (pH 6.2) in a 0.5 mm pathlength cell at 5°C . Spectra were taken in the presence of increasing concentrations of phosphate from 1-100 mM. Results were expressed as mean residue molar ellipticity $[\Theta]$ (deg $\text{cm}^2 \text{dmol}^{-1}$) which were calculated from the equation:

$$[\theta] = \frac{[\theta]_{\text{obs}} \times \text{MRW}}{10 \times l \times c} \quad (2)$$

where $[\Theta]_{\text{obs}}$ is the observed ellipticity in millidegrees, MRW is the mean residue molecular weight, l is the optical pathlength in cm, and c is the peptide concentration in mg/ml. The percentage of secondary structure was calculated by the Aviv Prosec V3.1 software which compares the protein spectrum with the reference spectra of 15 proteins of known secondary and tertiary structure [24]. The ellipticity of AK as a function of pH as well as phosphate was determined. All solutions were filtered through $0.2 \mu\text{m}$ membranes prior to use.

2.7 Results

2.7.1 Effect of Phosphate on Adenosine Affinity and Inhibition

The velocity of catalytic activity, enzyme affinity for adenosine and substrate inhibition by adenosine are greatly affected by the presence of Pi for both wild-type CHO and native BL AK. The data presented in Figs. 2.1-2.3 plot the initial velocity of AK, expressed as pmol AMP formed per minute, as a function of adenosine concentration. These figures suggest that at high concentrations of adenosine, the initial velocity reaches a plateau. These velocities would tend to zero for non-competitive substrate inhibition. Therefore, we chose to fit the data to an equation which does not discriminate between uncompetitive and competitive substrate inhibition [25;26]. This basic equation describes the velocity of an ordered bisubstrate reaction where the inhibitory substrate A (adenosine) is varied at a fixed concentration of non-inhibitory cosubstrate B (MgATP):

$$v = \frac{VA}{\left(K_M + A + \left(\frac{A^2}{K_i} \right) \right)} \quad (3)$$

where v is velocity, V is maximal velocity, A is the concentration of adenosine, K_m is the substrate concentration at which the velocity is half maximal and K_i is half the substrate concentration required to invoke complete inhibition.

The catalytic activity of CHO and BL AK is almost completely dependent upon the presence of Pi at pH 6.2 (Figs. 2.1 and 2.2). At concentrations of adenosine between 0.1 and 5 μ M, the velocity of CHO AK increases hyperbolically (Fig. 2.1). Above 5 μ M, adenosine begins to inhibit the enzyme. The addition of Pi serves to increase both the

enzyme affinity for adenosine and the velocity of the catalytic reaction. As the concentration of Pi is increased, the onset of substrate inhibition occurs at progressively higher concentrations of adenosine. At pH 6.2, substrate inhibition of BL AK is observed to be almost completely relieved by the addition of 10 mM Pi (Fig. 2.2). The effect of Pi on the kinetic constants of both the CHO and BL enzymes at pH 6.2 are shown in Table 2.1. CHO BL AK increases its affinity for adenosine, K_m from 9.5 to 0.7 μM , increases the half maximal concentration necessary to invoke substrate inhibition from 19 to 69 μM and more than doubles maximum velocity from 7.8 to 16.7 pmol of AMP formed per minute upon addition of Pi from 1 to 50 mM. The affinity of the BL enzyme for adenosine increases from 5.1 to 1.6 μM , K_i increases from 19 μM to greater than 1 mM and the maximum velocity is enhanced more than two-fold, from 8.6 to 22.7 pmol AMP formed per minute, upon the addition of Pi from 0.1 to 50 mM.

Figure 2.1 Effect of [Pi] on CHO AK K_m & K_i for adenosine at pH 6.2.

The initial velocity is plotted against increasing concentrations of adenosine in the presence of: 1 mM Pi (\blacktriangle); 2 mM Pi (\bullet); 3 mM Pi (\blacksquare); 5mM Pi (\triangle); 7 mM Pi (\circ); 10 mM Pi (\square); and 50 mM Pi (\blacklozenge). Activity assays were performed in 20 mM citrate buffer, 5 mM ATP and Mg^{2+} at 35°C.

Fig. 2.1

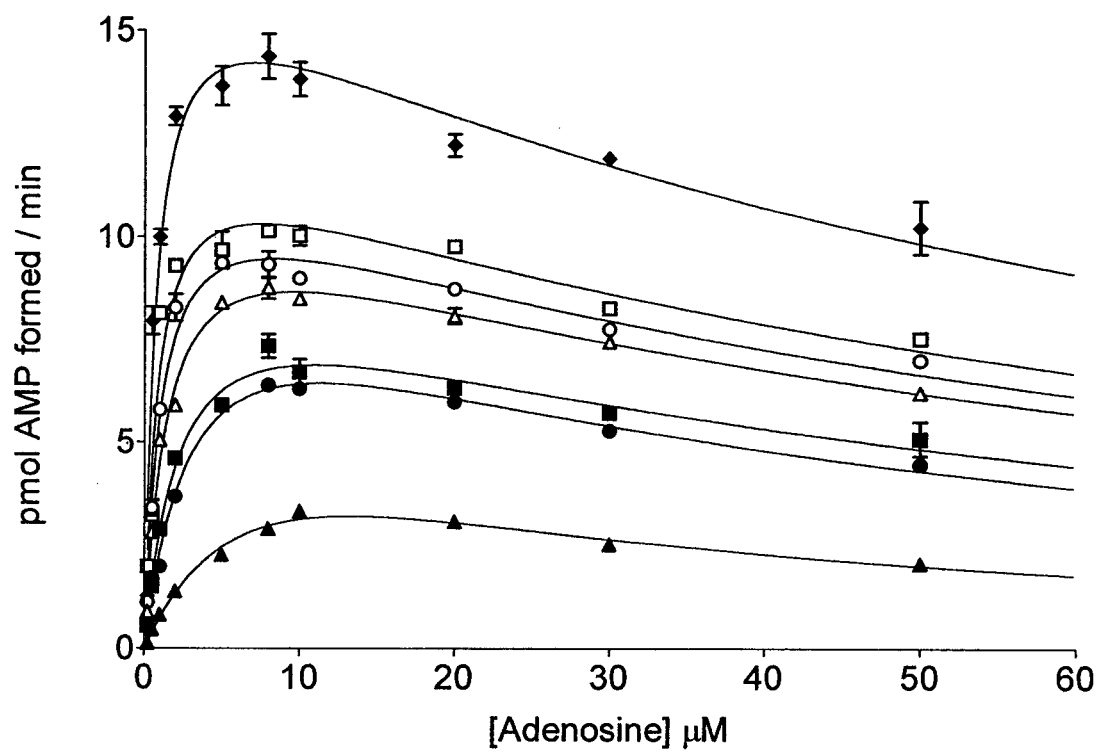


Figure 2.2 Effect of [Pi] on BL AK K_m & K_i for adenosine at pH 6.2.

The initial velocity is plotted against increasing concentrations of adenosine in the presence of: 0.1 mM Pi (\blacktriangle); 1 mM Pi (\bullet); 10 mM Pi (\blacksquare); and 50 mM Pi (\square). Activity assays were performed in 20 mM citrate buffer, 5 mM ATP and Mg^{2+} at 35°C.

Fig. 2.2

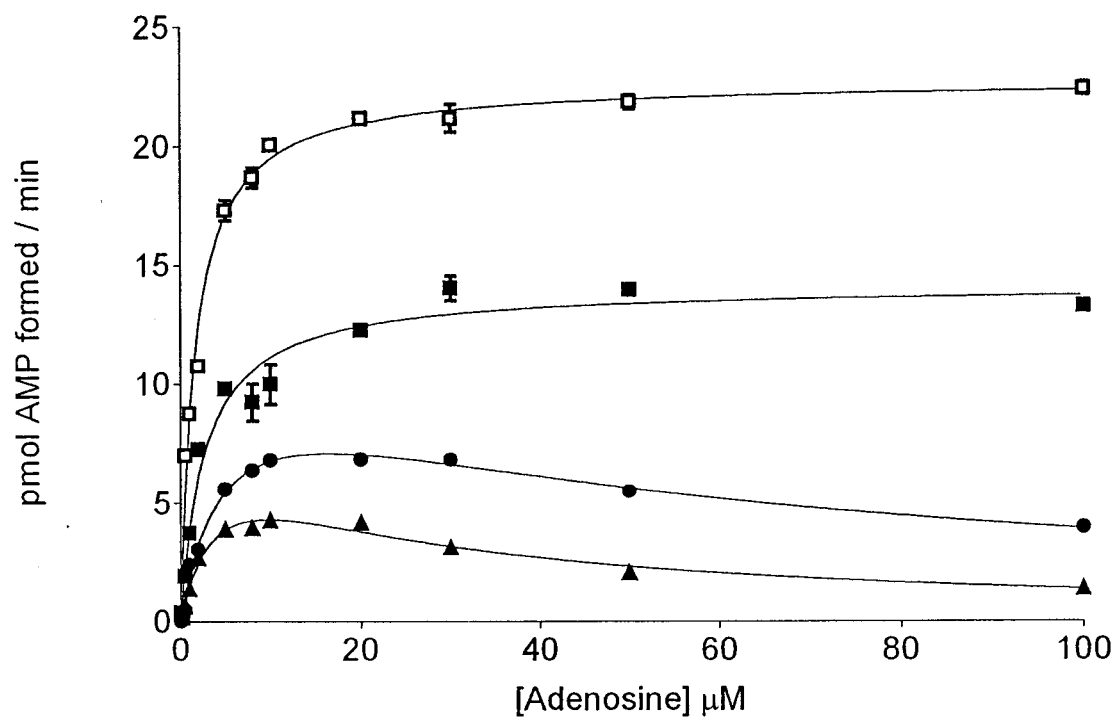


Figure 2.3 Effect of [Pi] on CHO AK and BL K_m & K_i for adenosine at pH 7.4.

The initial velocity is plotted against increasing concentrations of adenosine. (A) CHO AK in the absence of Pi (■) and in the presence of 20 mM Pi (▲); (B) BL AK in the absence of Pi (■) and in the presence of 20 mM Pi (▲). Activity assays were performed in 100 mM Tris buffer, 5 mM ATP and 5.75 mM Mg^{2+} at 35°C.

Fig. 2.3

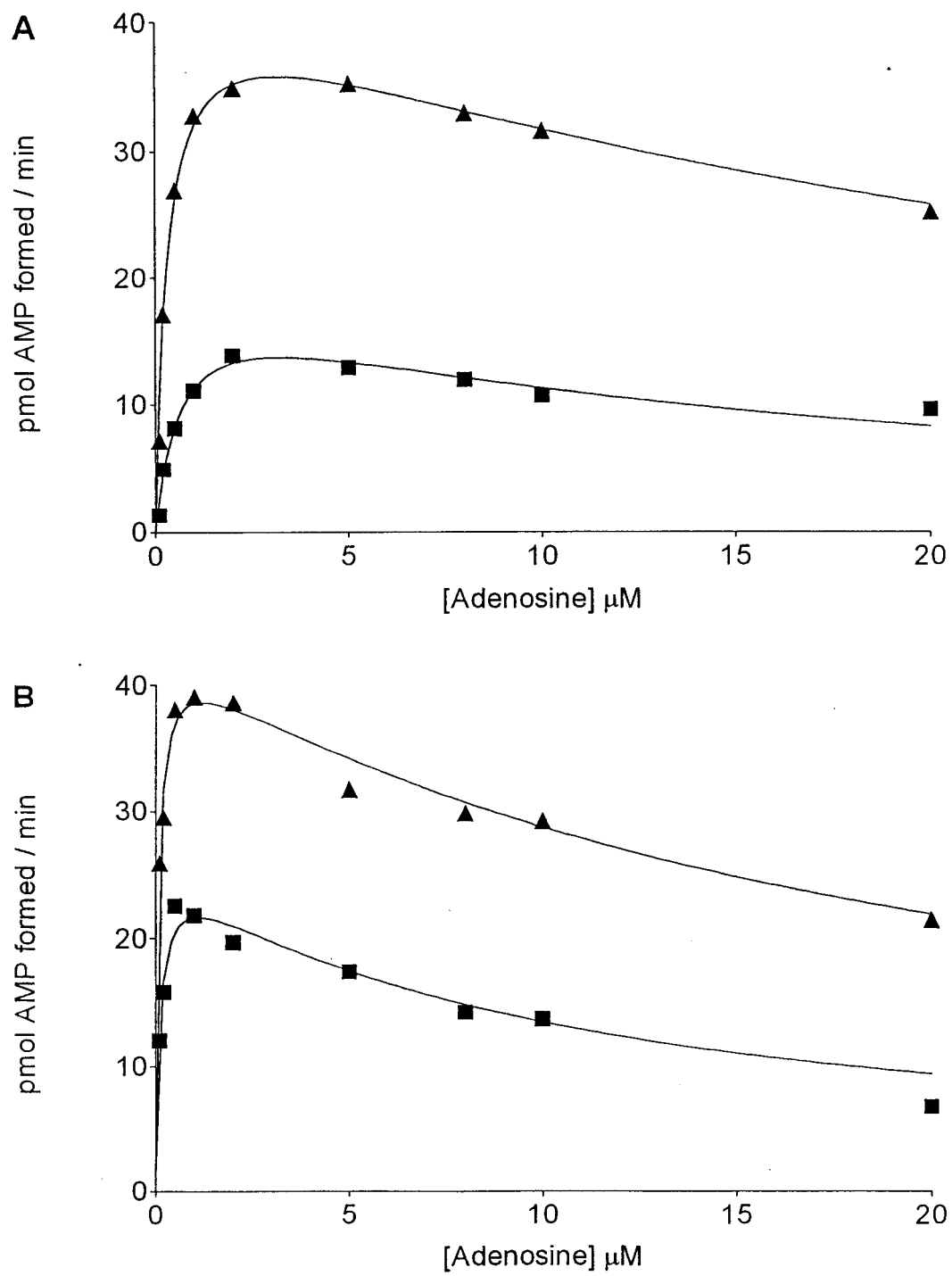


Table 2.1 Effect of [Pi] on the Kinetic Constants of AK for Adenosine at pH 6.2.

AK source	Pi (mM)	K_m (μM)	V_{max} (pmol AMP/min)	k_{cat} (s^{-1})	k_{cat}/K_m ($\text{M}^{-1}\text{s}^{-1}$)	K_i (μM)
CHO	1	9.5 \pm 2.6	7.8 \pm 1.4	0.46 \pm 0.08	4.88 $\times 10^4$	19 \pm 5.4
	2	2.7 \pm 0.50	9.0 \pm 0.80	0.53 \pm 0.05	1.98 $\times 10^5$	38 \pm 6.9
	3	2.1 \pm 0.40	9.1 \pm 0.80	0.54 \pm 0.05	2.57 $\times 10^5$	50 \pm 12
	5	1.5 \pm 0.20	11.1 \pm 0.60	0.66 \pm 0.03	4.39 $\times 10^5$	62 \pm 11
	7	1.1 \pm 0.20	11.5 \pm 0.70	0.68 \pm 0.04	6.21 $\times 10^5$	64 \pm 0.18
	10	0.86 \pm 0.23	12.4 \pm 1.1	0.74 \pm 0.07	8.18 $\times 10^5$	68 \pm 0.23
	50	0.74 \pm 0.19	16.7 \pm 1.4	0.99 \pm 0.08	1.42 $\times 10^6$	69 \pm 0.19
BL	0.1	5.1 \pm 1.3	8.6 \pm 1.3	0.51 \pm 0.08	1.00 $\times 10^5$	19 \pm 5.0
	1	4.5 \pm 0.90	11.2 \pm 1.0	0.67 \pm 0.06	1.48 $\times 10^5$	54 \pm 11
	10	2.6 \pm 0.70	14.0 \pm 1.1	0.83 \pm 0.07	3.20 $\times 10^5$	500 -
	50	1.6 \pm 0.20	22.7 \pm 0.9	1.35 \pm 0.05	8.42 $\times 10^5$	1000 -

Kinetic constants were obtained from data in Figs. 2.1 and 2.2.

Table 2.2 Effect of [Pi] on the Kinetic Constants of AK for Adenosine at pH 7.4.

AK source	Pi (mM)	K_m (μM)	V_{max} (pmol AMP/min)	k_{cat} (s^{-1})	k_{cat}/K_m ($\text{M}^{-1}\text{s}^{-1}$)	K_i (μM)
CHO	0	0.66 \pm 0.16	19.5 \pm 2.0	1.16 \pm 0.12	1.75 $\times 10^6$	15 \pm 3.6
	20	0.34 \pm 0.04	43.9 \pm 1.8	2.61 \pm 0.11	7.67 $\times 10^6$	29 \pm 4.0
BL	0	0.11 \pm 0.03	25.1 \pm 1.8	1.49 \pm 0.11	1.35 $\times 10^7$	13 \pm 1.5
	20	0.07 \pm 0.01	47.6 \pm 1.5	2.83 \pm 0.09	4.04 $\times 10^7$	22 \pm 2.5

Kinetic constants were obtained from the data in Fig. 2.3A and B.

Table 2.3 Effect of Phosphate on K_m^{ATP} at pH 6.2.

AK	Pi (mM)	K_m (μM)	V_{max} (pmol AMP/min)	k_{cat} (s^{-1})
CHO	0.1	0.36 \pm 0.12	1.4 \pm 0.1	0.08 \pm 0.01
	1	0.31 \pm 0.04	5.7 \pm 0.2	0.34 \pm 0.01
	10	0.27 \pm 0.05	12.1 \pm 0.6	0.72 \pm 0.04
	50	0.26 \pm 0.01	14.0 \pm 0.2	0.83 \pm 0.01
BL	5	1.45 \pm 0.22	7.4 \pm 0.4	0.44 \pm 0.02
	10	1.41 \pm 0.26	16.2 \pm 1.0	0.96 \pm 0.06
	50	1.37 \pm 0.17	40.3 \pm 1.5	2.40 \pm 0.09

Kinetic constants were obtained from data in Figs. 2.4 A and B.

At physiological pH, the catalytic activity of CHO and BL AK is greater than at the more acidic pH values and does not show complete dependence on the presence of Pi. However, V_{max} is doubled in the presence of 20 mM Pi. As well, the addition of 20 mM Pi increases the enzyme affinity for adenosine and increases the concentration of adenosine necessary to invoke substrate inhibition (Fig. 2.3 A and B). The effects of Pi on the kinetic constants for both enzymes at pH 7.4 are shown in Table 2.2. CHO AK doubles its affinity for adenosine from 0.7 to 0.3 μM , doubles its K_i from 15 to 30 μM and nearly triples maximum velocity from 19.5 to 43.9 pmol AMP formed per minute upon the addition of 20 mM Pi. BL AK increases K_i from 13 to 22 μM and the maximum velocity is raised from 25.1 to 47.6 pmol AMP formed per minute after the addition of 20 mM Pi.

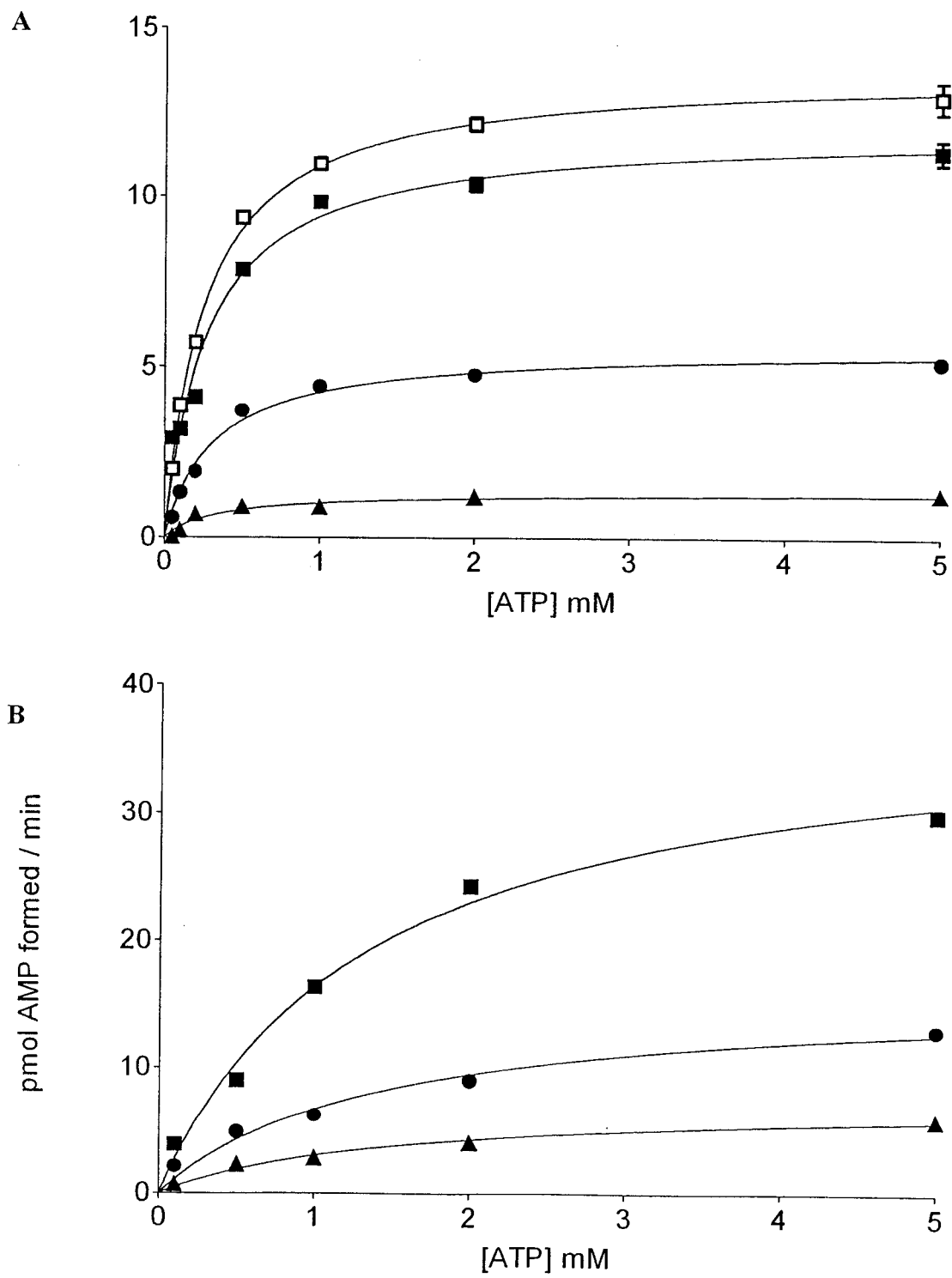
2.7.2 Effect of Phosphate on ATP Affinity and Inhibition

The addition of up to 50 mM Pi has very little effect on the affinity of both CHO and BL AK for ATP. However Pi does serve to increase the maximal velocity of activity for the enzymes (Fig. 2.4 A and B). The effect of Pi on the K_m^{ATP} and V_{max} for both enzymes at pH 6.2 are shown in Table 2.3. CHO K_m^{ATP} is slightly lowered, 0.36-0.26 mM, and V_{max} is increased, 1.4 to 14 pmol AMP formed per minute, consequent to increasing concentrations of Pi from 0.1 to 50mM. The affinity of the BL enzyme for ATP remains constant at 1.4 mM and V_{max} increases from 7.4 to 40.3 pmol AMP formed per minute.

Figure 2.4 Effect of [Pi] on CHO and BL AK K_m^{ATP} at pH 6.2.

The initial velocity is plotted against increasing concentrations of ATP. (A) CHO AK : 0.1 mM Pi (▲); 1 mM Pi (●); 10 mM Pi (■); and 50 mM Pi (□). (B) BL AK: 5 mM Pi (▲); 10 mM Pi (●); and 50 mM Pi (■). Activity assays were performed in 20 mM citrate buffer with 10 mM adenosine, Mg^{2+} free held fixed at 1.04 mM at 35°C.

Fig. 2.4



2.7.3 ΔK_m & ΔK_i in the Presence of Pi

The affinity constants of both CHO and BL AK for adenosine at pH 6.2 (data from Table 2.1) are plotted as a function of Pi concentrations (Fig. 2.5A and B). In the absence of Pi, the affinity of the BL enzyme for adenosine is 6-fold greater than that of the CHO enzyme, 5 μ M and 30 μ M respectively. However, BL and CHO AK are similar with respect to K_m in their Pi-bound forms, both at roughly 1 μ M. At pH 6.2, K_i also increases for both CHO and BL AK (Fig. 2.6). In their Pi free states, both enzymes show a $K_i(\text{min})$ of approximately 10 μ M. The maximum velocity increases hyperbolically for both enzymes in the presence of increasing concentrations of Pi (data from Table 2.1).

2.7.4 pH Studies

To determine the pH optimum of CHO AK activity in the presence of Pi, activity was assayed through a large range of pH values. Activity was monitored from pH 5.5 to 11.2 in the presence and absence of 20 mM Pi. The reaction was stopped after 15 min and the amount of radiolabelled AMP was counted. The presence of Pi serves to change the pH optimum for AK activity (Fig. 2.7). Activity in the absence of phosphate shows a narrow optimum at pH 6.5 to 7.5. The addition of 20 mM Pi serves to extend this range from 5.5 to 10. PO_4^{2-} is the dominant phosphate species found within this extended pH range.

Figure 2.5 Summary of Observed Affinity Constants of AK for Adenosine.

Calculated affinities are plotted as a function of phosphate concentration for: (A) CHO AK and (B) BL AK (data from Table 2.1).

Fig. 2.5

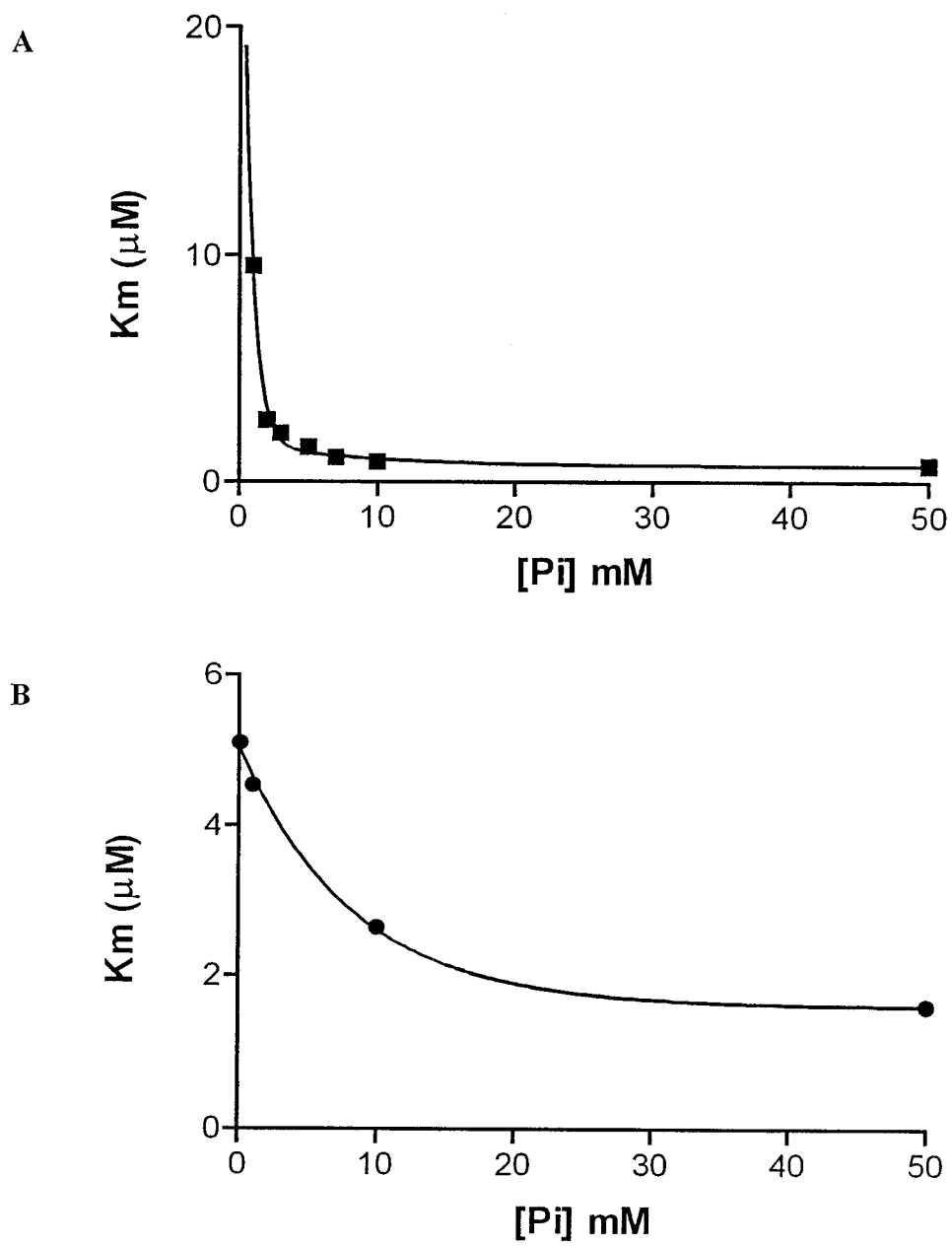


Figure 2.6 Summary of Observed Substrate Inhibition Constants of AK.

Calculated inhibition constants are plotted as a function of phosphate concentration for CHO AK (■) and BL AK (▲) (data from Table 2.1).

Fig. 2.6

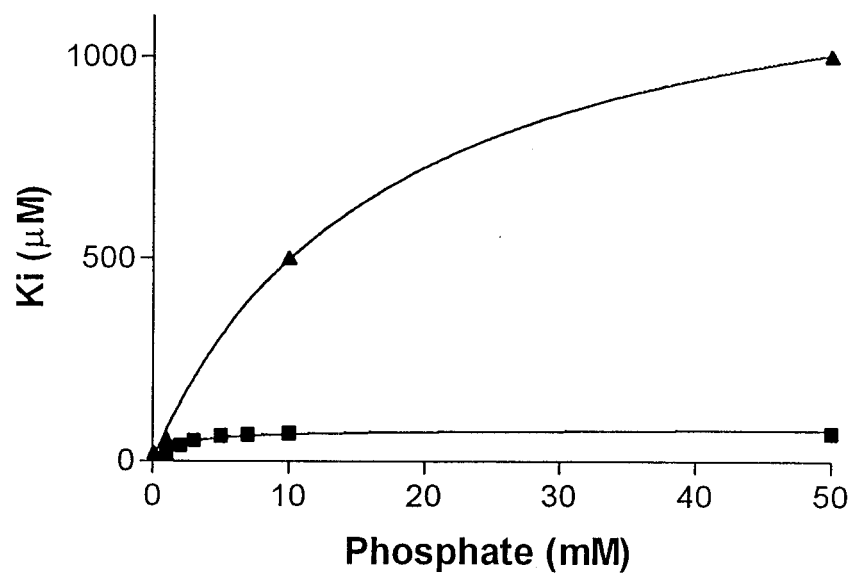


Figure 2.7 Effect of Phosphate on pH Optimum.

CHO AK assayed in the presence (●) and absence (□) of 20 mM Pi in 100 mM buffer at 35°C.

Fig. 2.7

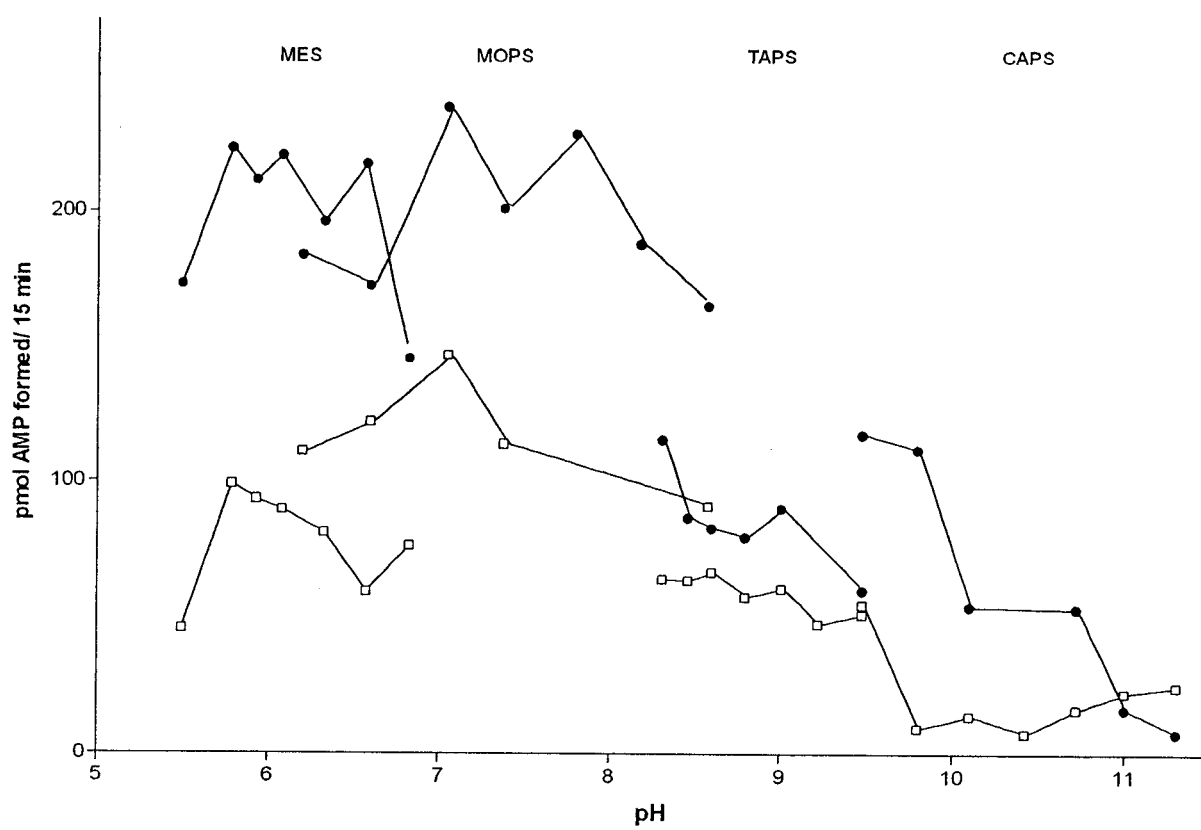


Figure 2.8 CD Spectra of CHO AK

CD spectra were obtained in the presence and absence of 100 mM Pi at: (A) pH 6.2 and (B) pH 7.4. CD spectra in the presence of 100 mM Pi (dashed lines) were recorded 15 min. after the addition of Pi.

Fig. 2.8

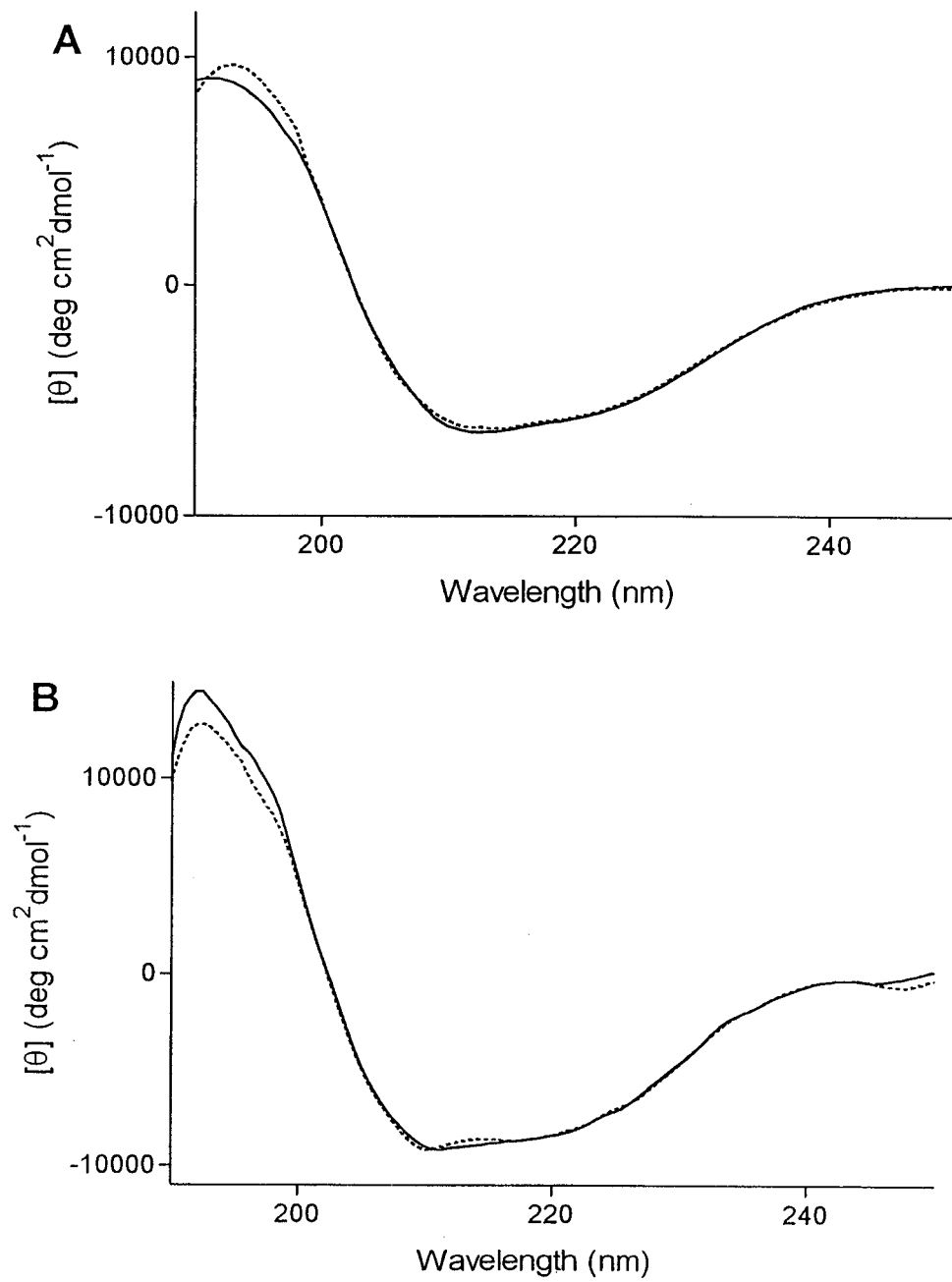
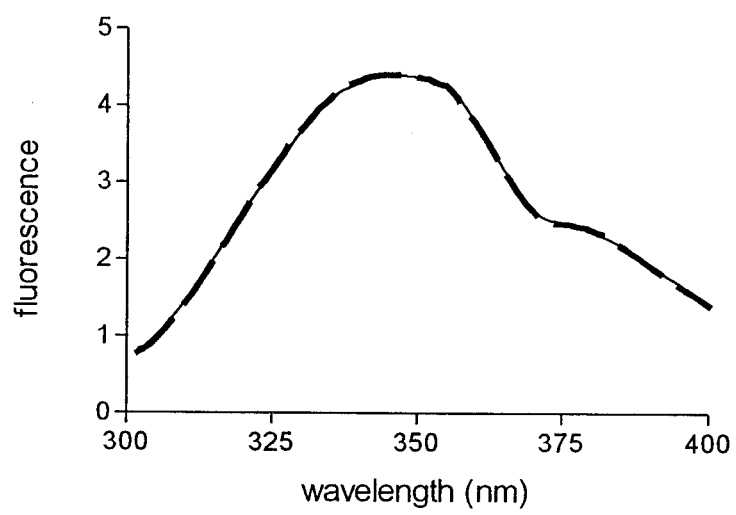


Figure 2.9 Fluorescence Emission Spectrum of CHO AK.

The resting enzyme is shown in thin, the protein/phosphate complex in thick dashed line.

Fig. 2.9



2.7.5 Structural Studies

CD spectra of CHO AK are shown in Fig. 2.8 A and B. Calculation of the secondary structure indicates that there is no significant change in the structure of AK upon addition of up to 100 mM Pi. At pH 7.4 (Fig. 2.8 A), the α -helical content is 12% and the β -structure content is 67% both in the presence (dashed line) and in the absence of phosphate (solid line). When the pH is decreased to 6.2 (Fig. 2.8 B), there is an increase in the overall spectral intensity. In this acidic environment, the percentage of α -helical content is enhanced to 17% and the β -structure is decreased to 63% for both the resting enzyme (solid line) and in the presence of Pi (dashed line).

The intrinsic fluorescence of CHO was also investigated (Fig. 2.9). AK exhibited broad fluorescence emission spectrum with a peak at 345 nm and a shoulder at 375 nm when excited at 280 nm. Unlike BL AK [27], neither the addition of adenosine, Mg/ATP²⁻ nor Pi caused any significant alterations in the intrinsic fluorescence emission spectrum of CHO AK.

2.8 Discussion

The focus of this study was to determine the effect of inorganic phosphate on the activity of adenosine kinase. Our data suggest that phosphate relieves substrate and H⁺ inhibition of the enzyme. AK is more sensitive to substrate inhibition at pH 6.2 than at pH 7.4. Catalytic activity appears to be almost completely dependent upon Pi at pH 6.2

and is greatly increased upon the addition of Pi at pH 7.4. The affinity of AK for adenosine also increases upon the addition of Pi for both pH values studied. The concentration of adenosine necessary to invoke substrate inhibition for both CHO and BL AK increases upon the addition of Pi. The BL enzyme exhibits almost complete relief of substrate inhibition in the presence of 50 mM Pi at pH 6.2. The pH optimum for activity is also extended in the presence of Pi. We have shown that not only is the catalytic activity of AK enhanced by Pi at physiological pH, but is required for activity at more acidic pH values.

The study of phosphokinase reactions are often complicated by substrate inhibition. If substrate combines with a form of enzyme other than at its resting state, the substrate can act as a dead-end inhibitor. This inhibition is generally linear. Substrate inhibition can also occur if high levels of substrate cause an altered order of reactant addition. This second type of substrate inhibition is generally hyperbolic [25] as shown AK in Figures 2.1, 2.2, 2.3A and 2.3B. We have shown that phosphate is efficient as a protector against substrate-induced enzyme inhibition. Pi may exert its effects by stabilizing the active site for substrate binding and product release.

Protonation of ATP by an acidic residue at the active site associated with phosphate-ester bond cleavage has been implied as being the most effective contribution to phosphoryl transfer. This general acid catalysis would also facilitate the leaving characteristics of the ligand by decreasing its pKa [28]. The deprotonation of this acidic residue may give rise to an abortive enzyme/substrate complex at high adenosine concentrations where adenosine may bind before the resting enzyme is regenerated. The

three-dimensional model of the adenosine kinase active site suggests that Asp 300 of the human enzyme may be involved in proton donation catalyzing the transfer of the γ -phosphate of ATP to adenosine [19]. Phosphate may exert its effects either by facilitating the reorganization of intrinsic or exogenous protons to regenerate the active protein or by increasing the nucleophilic characteristics of residues at the active site facilitating catalysis. Since Pi is not consumed in the catalytic reaction of AK [20;21], we assume that Pi interacts with one or two residues distal to the active site which act as proton donors/acceptors in the chemical reaction, as suggested by the increase in the enzyme affinity for adenosine in the presence of phosphate. Pi may then facilitate the removal of charges on the enzyme which cause an abortive enzyme/substrate complex. We have previously shown that other pentavalent ions such as arsenate and vanadate cause an increase in both activity and affinity of AK for adenosine [21]. We have yet to determine if these other pentavalent ions also relieve substrate inhibition.

Two other enzymes have been reported to show a similar dependence on Pi for activity, the bifunctional 6-phosphofructo-2-kinase (EC 2.7.1.105) and phosphoribosylpyrophosphate synthetase (EC 2.7.6.1) [29;30]. These enzymes share the common feature of phosphorylating a sugar derivative *via* ATP. Both 6-phosphofructo-2-kinase and AK are similar in that they share a three-layer $\alpha\beta\alpha$ sandwich core structure [19;31]. It is thought that Pi acts by evoking an active configuration for both 6-phosphofructo-2-kinase and phosphoribosylpyrophosphate synthase. Both CD and fluorescence spectra indicate that AK does not undergo a visible conformational change upon binding phosphate.

This study demonstrates that the factors which mediate AK activity are the concentrations of substrate and H^+ . We have shown that AK is more sensitive to substrate inhibition in an acidic environment. This substrate inhibition can be relieved by inorganic phosphate. A number of *in vivo* studies show that marked increases in the concentrations of adenosine [32-38] and H^+ [5;6] can be found in the heart and brain during prolonged episodes of ischemia. It has been suggested that excessive adenosine release may be a mechanism by which the heart and brain protect themselves from cell injury through an ischemic episode [32;39]. The protective effect of high adenosine concentration in the heart [32-34] and brain [35;36] is a result of the activation of cell surface adenosine receptors A1, A2a, A2b and A3. These studies suggest that endogenous inhibition of AK is a general mechanism for the amplification of adenosine under conditions of ischemia. Pharmacological inhibition of AK has been shown to further increase the myocardial and cerebral protective effects of adenosine [10;40]. Other studies indicate that if an ischemic episode is prolonged or severe enough, the concentration of inorganic phosphate is increased several-fold [4;6]. This response may be required to restore the pre-ischemic levels of adenosine through the salvage pathway.

2.9 Acknowledgements

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Chapter 3. Structure-Activity Studies On Mammalian Adenosine Kinase

Mary C. Maj, Bhag Singh and Radhey S. Gupta (2000) *Biochemical and Biophysical Research Communications*, 275, 386-393.

3.1 Preface

Earlier work in our lab had identified several unique properties of adenosine kinase, including the effects of pentavalent ions on the activity of adenosine kinase which was examined in Chapter 2. We had also found that the first 27 residues of the protein were necessary for activity. However, the importance of the first 27 amino acids had not been further examined. In 1997, McNally and coworkers had identified two isoforms of human adenosine kinase which differed only in the extreme N-terminus. Sequence analysis had also indicated that the greatest differences in the amino acid sequence of adenosine kinase among species is found at the far amino end of the protein. As such, we began to explore the importance of the first 27 residues of Chinese hamster ovary adenosine kinase. Systematic deletion of the residues of the N-terminal region were performed and the resulting proteins were tested for activity, K_m for both co-substrates, and thermal stability.

The results in this chapter revealed that removal of the first 13 amino acids had no significant effect on the activity of adenosine kinase. Further deletion of residues up to residue 27 results in a gradual decline in activity. Structural analysis of these proteins suggests that residues 23-27 are required for the formation of the first β -sheet structure of the protein.

As an efficient system for mutagenesis of the protein had been established, our studies were extended to include other mutations of adenosine kinase. C-terminal

deletions of the protein, as well as mutants of the putative catalytic base were constructed. Our results indicate that none of the C-terminal residues could be removed without substantial loss of activity. Replacement of the putative catalytic base D316 with either asparagine or glutamic acid resulted in nearly complete loss of activity.

I performed all the experimental work presented in this chapter with the exception of the construction of the original cDNA of adenosine kinase in both TOPO and pET expression vectors, a number of PCR reactions to create the mutated proteins and their subsequent ligation into TOPO vectors. These were performed by Dr. Bhag Singh.

Error values for the K_m adenosine, K_m ATP and $t_{1/2}$, not included in the published paper have been added to the data found in Figures 3.2 and 3.4. Secondary plots of thermal inactivation kinetics for both N- and C-terminal mutants are presented in section 3.10, Appendix A, at the end of the chapter.

3.2 Paper

Structure-Activity Studies On Mammalian Adenosine Kinase

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3.3 Abbreviations

AK, adenosine kinase; CHO, Chinese hamster ovary; TG, *Toxoplasma gondii*.

3.4 Abstract

The structure-activity relationship of Chinese hamster adenosine kinase (AK) was examined by making systematic deletions from the N- and C- terminal ends. The first 16 a. a. residues from the N-terminal end, which likely form a random coil, can be deleted without any effect on AK activity or stability. The successive removal of the next 11 residues, which stabilize the first β structure of the protein, leads to the progressive loss of AK activity from 100 to about 3%. The loss in activity is accompanied by increasing thermal instability and a slight increase in the K_m for adenosine. All deletions beyond the residue M28, which should cause disruption of the tertiary structure, are devoid of AK activity. The residues at the C-terminal end form a substructure involved in the stability of the 'adenosine 2 binding site' and removal of any residues results in significant loss of activity. Successive removal of the first 10 residues from this end causes progressive decrease in AK activity to approximately 2%, accompanied by a five-fold increase in the K_m for ATP, supporting the view that the 'adenosine 2' binding site located near the C-terminal end is the ATP binding site. All deletions beyond residue R348, which forms two salt bridges with the ATP binding site, are inactive. Site-directed replacement of an aspartic acid residue (D316), which is postulated to function in the transfer of the γ -phosphate of ATP to adenosine by either asparagine or glutamic acid, leads to complete loss of activity, supporting the proposed role of D316 as the catalytic base.

3.5 Introduction

The enzyme adenosine kinase (ATP:adenosine 5'-phosphotransferase, AK;EC 2.7.1.20) which catalyses the phosphorylation of adenosine to AMP [1-5] plays a key role in the regulation of intra-and extra-cellular levels of adenosine [6;7]. Elevated levels of adenosine in heart [8-10] and brain [11;12] are associated with the attenuation of ischemic injury, and in animal models, the inhibition of adenosine kinase (AK) has been shown to markedly increase the therapeutic effect of adenosine [11-14]. This has led to much interest in understanding the physiological functions of AK as well as to develop selective inhibitors of the enzyme with therapeutic potential. Although AK has been studied in the past from a variety of sources [2-5;15], many details regarding the mechanism of AK activity, including dependence of its catalytic activity upon pentavalent ions [16;17] and the order of substrate binding and product release [3;5], remains to be determined.

In recent years, the cDNA/gene for AK has been cloned and sequenced from a variety of organisms including humans and other animals [17-19], to plants [20;21], and eukaryotic microorganisms [22-25]. There is greater than 95% amino acid sequence similarity among AK from mammalian sources viz., human, rat, mouse and Chinese hamster, and the mammalian AK exhibit greater than 55% sequence similarity to the enzymes from plant and eukaryotic microorganisms. Recently the crystal structure of AK

has been solved from human [26] as well as *Toxoplasma gondii* [27]. These developments have set the stage for carrying out detailed structure-function studies on the enzyme.

In earlier work from this laboratory, AK cDNA was cloned from human, rat, mouse and Chinese hamster cells [17] and a large number of mutants of CHO cells which affect AK in novel manners were isolated and characterized [28-31]. Some of these mutants were shown to involve gross structural alterations in the AK gene [17]. A number of novel biochemical characteristics of AK, including pentavalent ion dependency of its catalytic activity [16;32], were also reported and the biochemical basis of this was investigated [33]. To further understand the roles of different structural regions of AK, we have now carried out systematic deletions of residues from the N- and C-terminal ends of AK. After expressing these mutants in *E. coli*, the enzymatic and other characteristics of the mutant proteins were studied. The changes in the activities/properties of these mutants have been correlated with the structure of the enzyme and they provide valuable information regarding the residues that are critical in maintaining structural stability of AK in ATP binding. In addition, site-directed mutagenesis was used to examine the function of an aspartic acid residue, which is thought to participate in the catalytic mechanism of adenosine kinase [26].

3.6 Methods and Materials

3.6.1 Deletion and Site-directed Mutants of AK

The N- and C-terminal deletion mutants were generated from full length CHO AK in a plasmid vector by designing PCR primers to specifically remove the desired numbers of N- or C-terminal amino acid residues. The AK in the plasmid is flanked by *NdeI* and *BamHI* sites and these sites were incorporated in the primers for cloning purposes. For C-terminal deletions, the stop codons were also incorporated in the primers. The PCR amplified fragments were initially cloned into a TA-vector and after their sequencing, the fragments were excised with *NdeI* and *BamHI* and subcloned into the expression vectors pET 15b (for C-terminal deletions) or pET22b (for N-terminal deletions) (Novagen). Site directed mutagenesis of aspartic acid residue (D316) was carried out by the use of 'Quikchange' site-directed mutagenesis kit (Stratagene). Specific overlapping primers in the opposite orientation carrying the appropriate changes were designed and after their annealing to the full length AK cDNA in pET15b, thermal extensions of the DNA strands was carried out using the *Pfu* DNA polymerase. After temperature cycling, the DNA was treated with *DpnI* to digest the parental DNA strands and the mutant plasmid DNA was transformed into BL-21 (DE3) cells. The respective changes in the mutants were verified by DNA sequencing. Expression of the recombinant protein in *E. coli* was induced by the addition of 0.1 mM IPTG and the expression was allowed to proceed overnight at room temperature. The expression levels of different deleted proteins was comparable in these experiments. The cell extracts were made in 20 mM Pi buffer pH 7.8 and 100 mM NaCl

using French press. All cell extracts were normalized to 0.5 mg/ml of final protein concentration. The protein from mutations at D316 were nickel column purified to >95% homogeneity. All protein samples were stored at -20°C in lysis buffer containing 0.1 mM DTT, 0.1 mM EDTA and 5% glycerol.

3.6.2 AK Activity Assays

[2,8-³H] adenosine (30.1 Ci/mmol) was purchased from Dupont Canada Inc. All other chemicals were of analytical reagent grade. The standard reaction mixture for AK activity determination contained 10 μM adenosine, 1 mM ATP, 2.46 mM MgCl₂ and 100 mM phosphate buffer (pH 7.4) in a final volume of 300 μl. For *K_m* determination of ATP, adenosine concentrations were held fixed at 10 μM and free Mg²⁺ at 1.5 mM, whereas for *K_m* determination of adenosine, ATP and MgCl₂ concentrations were held fixed at 1 mM and 2.46 mM respectively. The AK activity assays were normally performed at 37°C and initiated by the addition of enzyme to the reaction mix. Aliquots of 50 μl were withdrawn at various times in the linear reaction range and added to 1 mL of ice-cold 100 mM lanthanum chloride to precipitate phosphorylated product. The precipitate was collected, washed and radioactivity representing AMP formation was counted and analyzed as previously reported [17;31;33].

Thermal inactivation was performed by incubating the enzyme samples at 45°C. At various time intervals, aliquots were withdrawn and the samples were placed on ice for use in initial velocity determination. The AK activities at different times were plotted as % of control and from these plots the half-life for inactivation of activity to 50% of the

control was determined.

3.6.3 Structural Analysis

The three dimensional coordinates of adenosine kinase by Mathews *et al.* [26] were used for the modeling studies with the mutants. Close contacts between side chains of the mutated regions with the entire protein were calculated with Swiss-Pdb Viewer version 3.5 modeling software.

3.7 Results

The AK from CHO cells consists of 361 residues (Fig. 3.1). In our earlier work, where the cloning of CHO AK was described [17], due to an unrecognized sequence compression, an upstream translation initiation codon from which AK is translated was not recognized and the coding sequence was indicated to be slightly shorter (334 a.a. long) than what it actually is. Similar to CHO cells, AK from rat is also 361 residues long. In contrast to the rodent species, in humans two different isoforms of AK have been described [18;19]. In addition to the long form (362 a.a.) which corresponds closely to the form found in the rodent species, a shorter form (345 a.a.) which lacks about 17 residues near the N-terminal end had been described (Fig. 3.1). This shorter form is postulated to arise by alternate splicing of the AK mRNA [18;19]. Previous studies indicate that both the long and the short forms are active with similar substrate binding affinities [19].

3.7.1 N-terminal Deletions.

To understand the roles of the residues near the N-terminal end, systematic deletions which removed increasing numbers of amino acid residues from the N-terminal ends were constructed (Fig. 3.2A). After expression of these deletions in *E. coli*, the AK activity of the resulting proteins were determined. The first 3 deletions which removed 3, 8 and 13 residues after the initiating methionine had no significant effect on AK activity or other characteristics of the expressed protein. However, as further deletions were carried out which removed between 17 to 25 residues from the N-terminal end, a gradual but marked effect on AK activity was observed (Fig. 3.2B). A further deletion of 2 amino acids which removed the Gly and Met residues at positions 27 and 28 (deletion N6), led to nearly complete loss of activity (<0.5% of wild-type). To understand whether the loss of AK activity was associated with a change in the binding affinity for any of the two substrates, the *K_m* for ATP and adenosine for various active deletions were determined. As seen, the *K_m* value for ATP does not change significantly for the various active deletions (0.20 mM for the wild-type vs. 0.23 for the largest active deletion N5). However, there is a slight but gradual increase in the *K_m* for adenosine (1.80 μ M for N5 protein as compared to 1.05 for the wild-type) as deletions extended into the AK structure, suggesting that there is some perturbation of the adenosine binding site.

Figure 3.1 Sequence Alignment of AK.

Sequences are from CHO cells with human long (L), human short (S) and rat AK.

Identical aligned residues are annotated with a star and similar residues with a colon. The position of aspartic acid 16 is marked with an arrowhead. The conserved NPLLD region is outlined. Genbank Accession Numbers for the AK sequences are: CHO AK, P55262; Rat, Q64640; Human long (L) and short (S) forms, P35263.

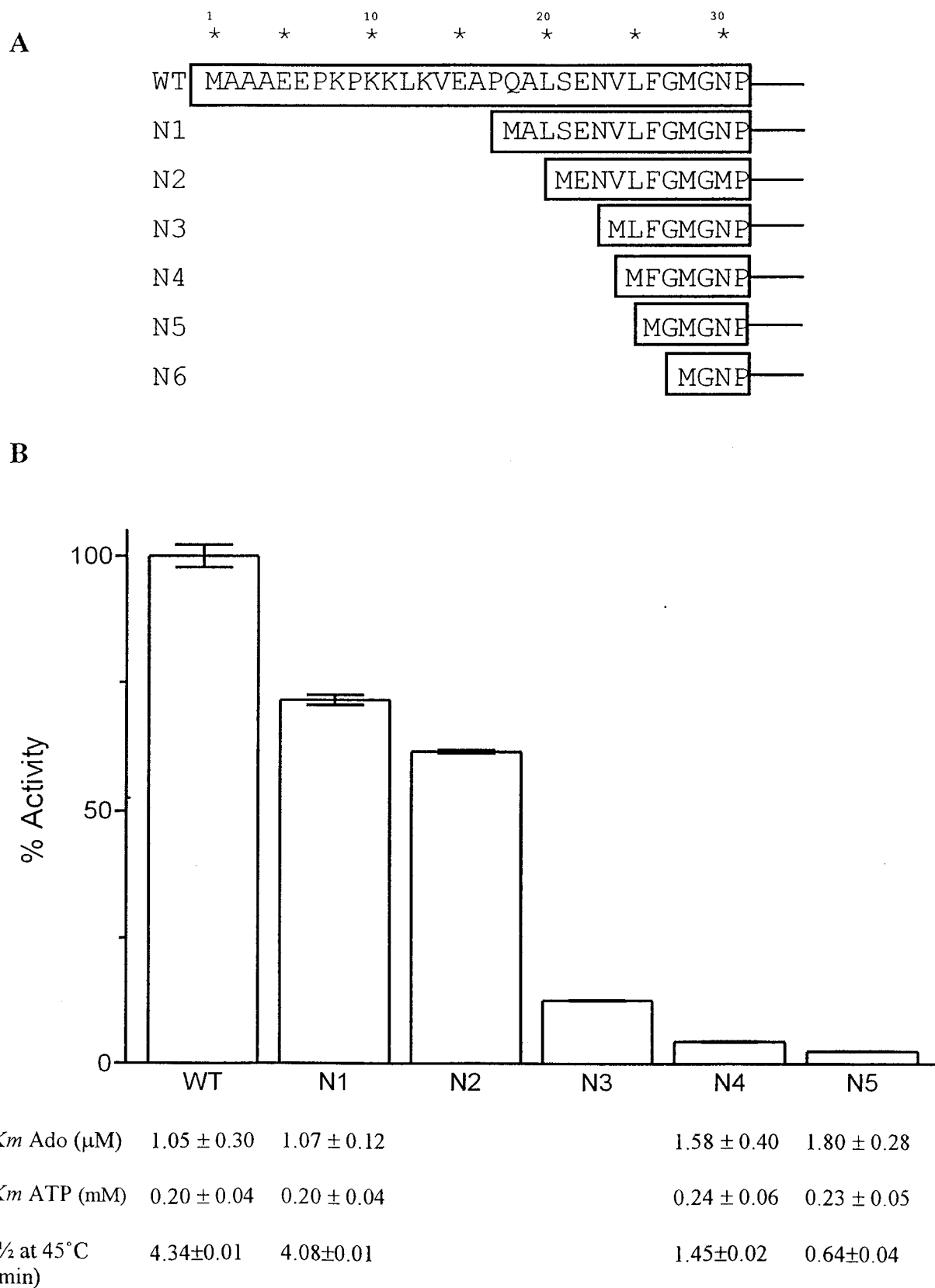
Since the deletions which led to a major loss of AK activity showed little effect on the binding of substrates, the possibility that the loss of AK activity was due to a structural effect on the protein was investigated. To examine this, the thermal stability of mutant enzymes was determined at 45°C. The results of these studies are also summarized in Fig. 3.2B. From the half-lives of residual AK activity from different mutants, it is clear that as the amino acid residues which lead to loss of AK activity are deleted, the resulting enzyme becomes more and more thermolabile. These results provide evidence that the amino acid residues at the N-terminal end (i.e., between positions 16 to 26) whose deletion lead to loss of AK activity, are involved in the structural stabilization of AK.

To understand the structural significance of these results, the crystallographic data for the human AK was examined. The sequence data for CHO AK, which shows over 95% sequence similarity to human AK, and was threaded into the structure [26]. Figure 3.3 is a ribbon representation of the N-terminal region of AK from CHO cells. Residues 1 to 18 are not assigned structure as they are not found in the human isoform for which crystallographic data is available. The *T. gondii* protein is 6 residues longer than human short form at the N-terminal end, however the first 10 residues were not resolved in its structure [27]. It is likely that the N-terminal residues upstream of $\beta 1$ (Fig. 3.3) are not characterized by regular hydrogen bonding patterns and exists as random coil structures. This is typical of protein terminal arms. The first residue which is involved in hydrogen bonding is N23 (corresponds to N7 in the short form of human AK). N23 forms a close

Figure 3.2 Effect of N-terminal Deletions (A) on AK Activity and Biochemical Characteristics (B).

The various N-terminal deleted proteins are shown (A). The results for three additional deletions, which removed 3, 8 or 13 residues from the N-terminal without any effect on activity are not shown. The AK activity in (B) is relative to the full length protein. The activity measurements for all deletions were made on three independent samples and their averages and standard errors are shown. The half-lives at 45°C of AK from different mutants were determined as described under Materials and Methods.

Fig. 3.2



contact with T101 within $\beta 5$ and D125 within $\beta 6$. N23 also forms a contact (via atom ND2) with the atom NZ of K177 located in the loop between $\alpha 5$ and $\beta 9$. The deletions N1 and N2 retain these regular structures and are the most active of the N-terminal mutants. However, in going from deletion N2 and N3, where these regular structures are not retained as a result of the deletion of residue N23, the AK activity dramatically decreases to less than 13%. The data suggests that the contacts of N23 with $\beta 5$, $\beta 6$ and K177, are necessary to form the β -sheet core of the tertiary $\alpha\beta\alpha$ sandwich structure of the protein.

Residues L25, F26, G27 and M28 are part of the first β structure of the protein (Fig. 3.3). As these residues are sequentially deleted in mutants N4, N5 and N6, the regular backbone hydrogen bonding between $\beta 1$ and $\beta 5$, and $\beta 1$ to $\beta 9$ is disrupted. This causes further decrease in AK activity and thermal stability. The proximity of the $\beta 1$ structure to some of the residues (e.g., N30) which form the adenosine binding site also explains the observed effect of deletion in this region on the binding of adenosine.

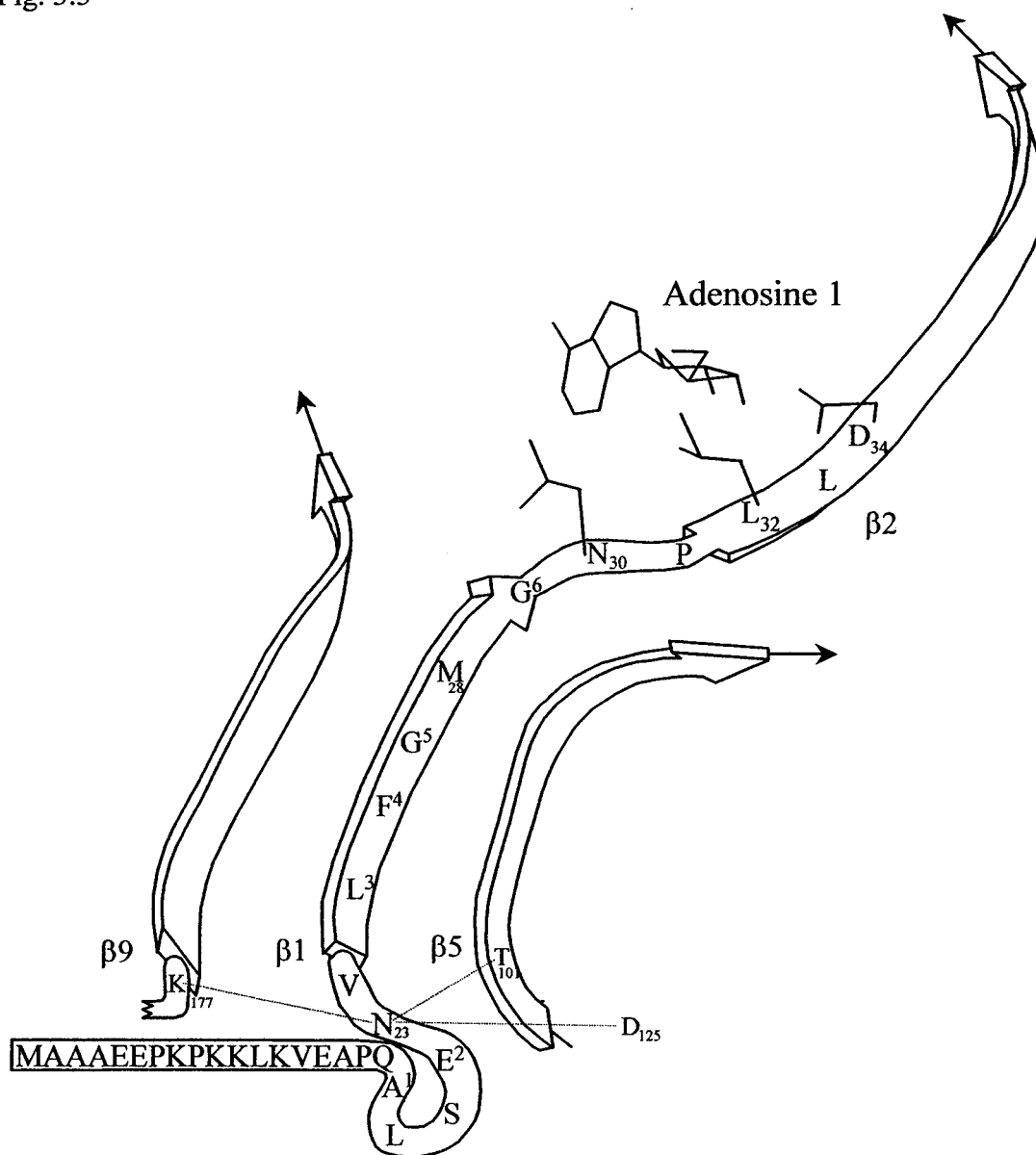
3.7.2 C-terminal Deletions

Systematic deletions were also made from the C-terminal end and their AK activities and other properties were examined (Fig 3.4). Mutant C1, which deletes only two residues, retains only 41% activity of the wild-type enzyme. The deletions C2, C3, and C4, which remove 5, 7 and 10 residues from the C-terminus, show 29%, 13%, and 2% activity compared to the full length protein. Further deletions from the C-terminal end

Figure 3.3 A Ribbon Diagram Depicting the N-terminal Region of AK.

This view shows the secondary and tertiary structural interactions of the residues present at the N-terminal end. The close proximity and interaction of $\beta 1$ to $\beta 9$ and $\beta 5$ is shown. Residues between and among $\beta 1$ and $\beta 2$, which form the 'adenosine 1' binding site are also shown. The first 19 residues are not assigned secondary structure and are assumed to be random coil. The numbers in subscript indicate the position of the residue in the CHO AK sequence, whereas the superscripted numbers correspond to the beginnings of the various deletions (Fig. 3.2A). The figure is based on the crystallographic data of Mathews *et al.* 1998 [26].

Fig. 3.3



as in mutants C5 and C6 lead to nearly complete loss of activity (<1% activity). The C-terminal mutants show only a small change in the Michaelis constant for adenosine (K_m values of 1.05 μM for wild-type vs. 2.60 μM for the largest active deletion C4).

However, a much greater effect is observed in the K_m for ATP for these mutants (0.19 mM for the full-length AK vs. 1.01 mM for the C4 mutant), suggesting that the residues at the C-terminal end are important for ATP binding.

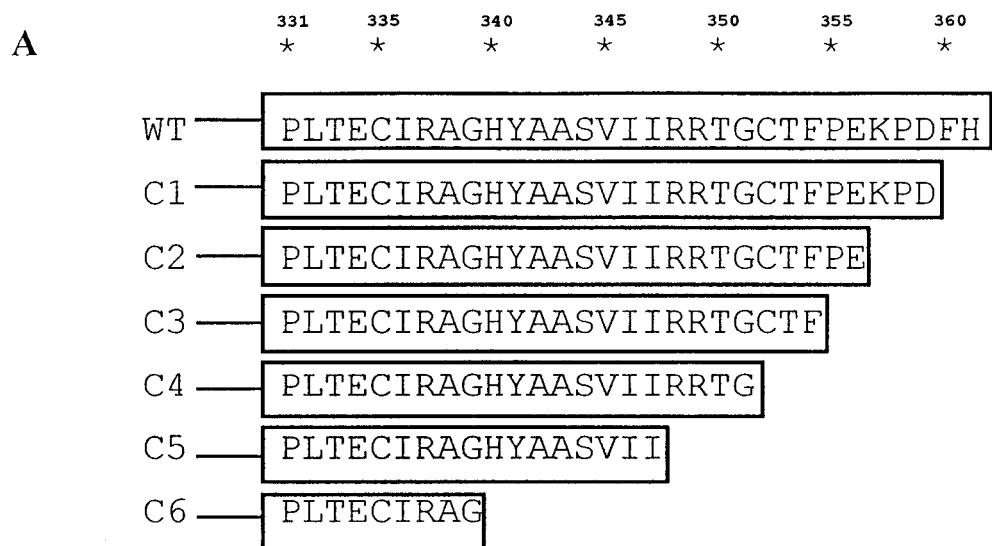
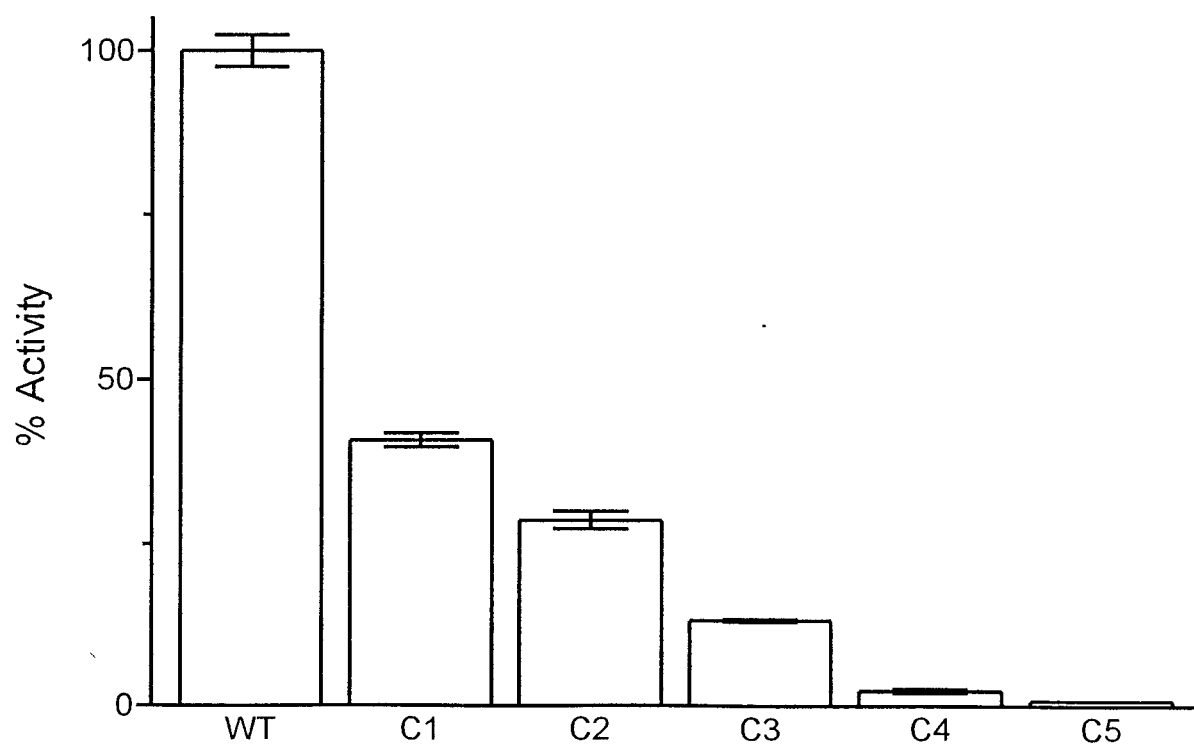
Thermal inactivation kinetics of the C-terminal deletion mutants were also determined at 45°C (Fig. 3.4B). From the $t_{1/2}$ values given in this figure, it is clear that similar to the N-terminal mutants, deletions from the C-terminal end also affected the stability of the enzyme. This provides evidence that the residues at the C-terminal end, in addition to their effect on the ATP binding, are also important in stabilizing the tertiary structure of the protein.

A ribbon diagram of the C-terminal region is shown in Fig. 3.5. The residues at this end form local secondary structures that are part of the network for the adenosine 2 or AMP-PCP binding site [26;27]. As different residues are deleted from the C-terminal ends, the contacts which form the network for this binding site are gradually disrupted leading to an increasing loss of AK activity. The carbonyl oxygen of the terminal histidine residue forms close contacts with R337 at atom NH2. The removal of the terminal histidine residue, as seen for the mutant C1, results in a 59% decrease in activity.

Figure 3.4 Effect of C-terminal Deletions (A) on AK Activity and K_m (B).

The activity measurements for all deletions were made on three independent samples and their averages and standard errors are shown.

Fig. 3.4

**B**

<i>K_m</i> Ado (μ M)	1.05 \pm 0.24				2.60 \pm 0.27	
<i>K_m</i> ATP (mM)	0.19 \pm 0.02	0.28 \pm 0.03	0.59 \pm 0.08	0.68 \pm 0.06	1.04 \pm 0.11	
t1/2 at 45°C (min)	4.32 \pm 0.02	2.82 \pm 0.03	1.46 \pm 0.03	1.42 \pm 0.05	0.89 \pm 0.03	0.76 \pm 0.07

The backbone nitrogen atom of D359 forms a close contact with Y341 at the OH atom. This contact is required to form a bend at E356 and K357. The removal of this bend, as seen in the mutant C2, results in a 71% decrease in activity and 66% decrease in stability. P355 at the carbonyl oxygen forms close contact with NE1 of W95. Mutant C3 in which this contact is lost shows a further decrease in activity. Residues R349, T350, G351 and C352 form a bend, with close contacts made between the carbonyl oxygen of T350 and OG1 of T353. Disruption of this bend as seen for mutant C4 results in an additional decrease in activity (residual activity only 2% of wild-type). Residue R348 forms two salt bridges with D302 located in the large P-loop region as shown in Fig. 3.5. The removal of this residue results in complete loss of AK activity indicating that the interaction of R348 with D302 is essential in the formation of the ATP binding site.

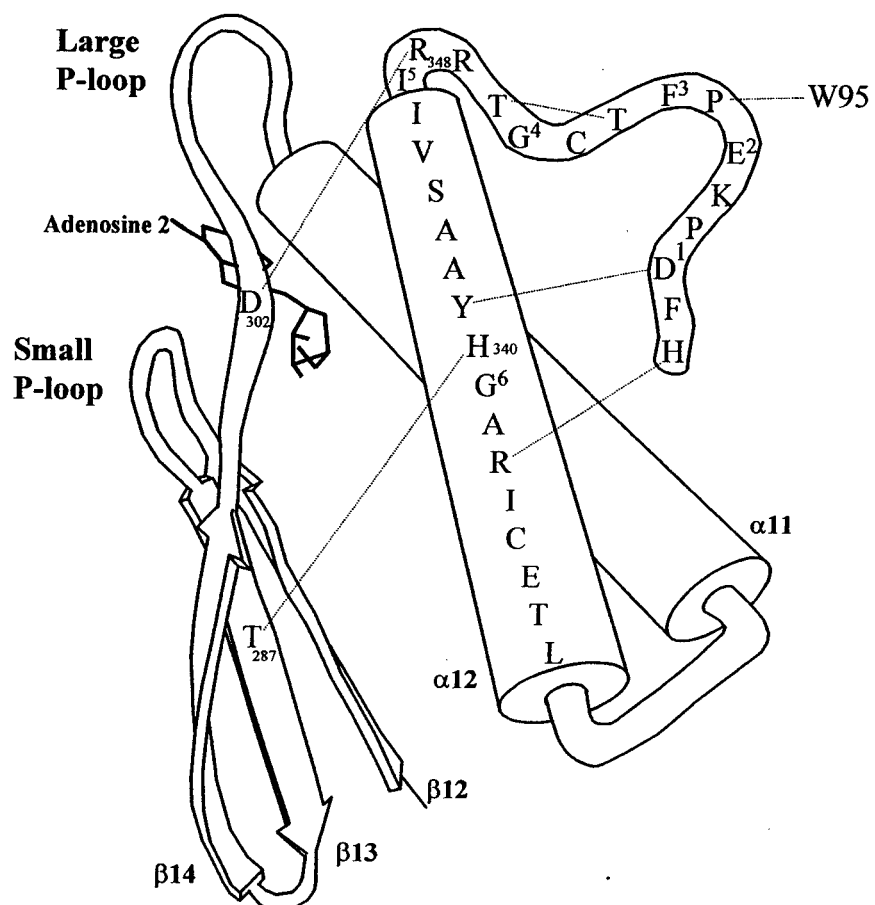
3.7.3 Active Site Mutants

It has been suggested that an aspartic acid residue is responsible for the deprotonation of adenosine at the 5' hydroxyl which initiates the transfer of the γ -phosphate of ATP [26;27]. Based on the location of this residue in 3D space, this residue is suggested to be D300 in the short form of human AK, D318 for *T. gondii* AK, and D316 in CHO AK (Fig. 3.6). An aspartic acid residue is found in the structure of bacterial ribokinase which occupies similar 3D space near the 5'-hydroxyl of ribose [34]. By means of site-directed mutagenesis, we have mutated this residue to asparagine and

Figure 3.5 Ribbon Diagram for the C-terminal Region of AK.

Close contacts that are made by the residues in this region which aid in the formation of the 'adenosine 2 binding site' shown as the large and small P-loops. The numbers in subscript indicate the position of the residues in the CHO AK sequence. The superscripted numbers refer to various deletions. This figure is based on the crystallographic data of Mathews *et al*, 1998 [26].

Fig. 3.5



glutamic acid. Both these substitutions led to nearly complete loss of activity. However, the recombinant proteins were purified and their specific activity was determined. The specific activity for D316N and D316E was found to be 2.44 and 3.37×10^{-6} mmoles AMP formed/min/mg compared to 1.27 mmoles AMP formed/min/mg for the wild-type protein. Computer modeling of the mutant D316E suggests that the loss of activity is mainly due to steric interference caused by the additional carbon unit in the glutamic acid residue. The data suggests that D316 is a required catalytic residue at the active site.

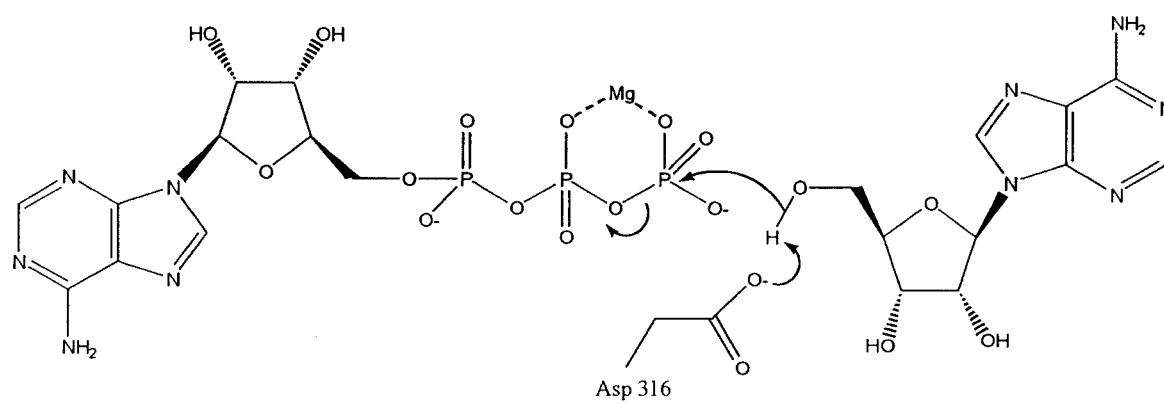
3.8 Discussion

Results presented here provide insight into the structure-activity relationship for mammalian adenosine kinase. The first useful insight obtained relates to the structural and functional roles of the residues present near the N-terminal end of the protein. The primary structure of AK at the N-terminus shows considerable variation in different isoforms as well as in different species. Two different isoforms of AK have been cloned and sequenced from human tissues [18;19], which are identical in sequence as well as biochemical properties [19] except for sequence variation in the first 15-20 amino acids near the N-terminus. These two forms are postulated to arise from a single AK mRNA by differential splicing. Evidence derived from somatic cell hybrids also suggest the existence of more than one AK isoform in human and mouse cell lines [35]. Although

Figure 3.6 Proposed Catalytic Role of the D316 Residue

D316 acts as a catalytic base which deprotonates the 5'-hydroxyl of adenosine to initiate the transfer of the γ -phosphate of ATP to form AMP. The figure is adapted from Mathews *et al.* 1998 [26].

Fig. 3.6



these studies indicate that the N-terminal region can tolerate some structural variation, the physiological significance of this variability or how far does it extend into the sequence remain unclear. The functional activity of various systematic deletions that we have made from the N-terminal end now clarifies this issue. Our data indicate that the first 16 a.a. residues of AK (i.e. MAAAEPPKPKKLVKA), which likely form a random coil, can be deleted without any affect on either AK activity or stability. Most of the previously observed variability in AK sequence either among different species or within a species is also restricted to this region [17-22;24]. As further deletions of AK sequence is carried out, progressive decline of AK activity, accompanied by decreased thermal stability of the residual enzymatic activity, is seen for the next 8 residues. The K_m of the residual activity is unaltered for ATP but is increased about 1.7-fold for adenosine. All of these changes are readily understood in terms of the structural models of AK, where the residues 21-23 participate in a hydrogen bonded turn and contacts of N23 with T101 in β_5 and K 177 in β_9 , are important in the formation of the β -sheet core of the $\alpha\beta\alpha$ sandwich structure of the protein. The next three residues viz., L25, F26 and G27, which are conserved in most AK sequences, are crucial in the formation and stabilization of the first β -structure of the protein [26;27]. As these three residues are sequentially deleted, the size of the β structure is reduced and the regular main chain hydrogen bonding between β_1 to β_9 and β_5 is increasingly disrupted. The proximity of these residues to some of the residues involved at the adenosine binding site also accounts for the observed reduced binding affinity for adenosine. All AK deletions beyond M28 would involve destabilization of the tertiary structure and are devoid of activity.

Studies of the C-terminal deletions indicate that none of the residues from this end could be deleted without a significant loss of activity. This is because the residues at this end form local secondary structures involved in forming the adenosine 2 or AMP-PCP binding site [26;27]. As the first 10 residues from the C-terminal end are deleted, the contacts which form the network for this binding site are gradually disrupted leading to an increasing loss of AK activity. The residue R348, which forms two salt bridges with a residue in the large P-loop region of AK, has been identified as a critical residue for activity and all deletions beyond it are totally devoid of activity. The deletion of residues from the C-terminal end leads to a gradual increase in the K_m for ATP up to greater than 5-fold, with a much smaller effect on the K_m for adenosine. This suggests that the interaction of residues at this end, particularly that of R348 with D302, are important for the formation of the ATP binding site.

In the reaction catalyzed by AK, an amino acid residue which functions as a catalytic base has been proposed to initiate the adenosine kinase transfer of phosphate from ATP to adenosine [26]. Biochemical studies have shown that there is a large decrease in the activity of AK below pH 5.5, suggesting the existence of a catalytic aspartic acid residue [2]. The 3D model of both *T. gondii* and human AK indicate a hydrogen bond between the 5'-hydroxyl group of adenosine (the phosphate acceptor) and the OD2 carboxylate of an aspartic acid residue (corresponding to D316 in CHO AK) [26;26;27]. The data implies that D316 functions as the general base which deprotonates the 5'-hydroxyl for attack on the γ -phosphate of ATP. The transfer of the phosphoryl group from ATP to adenosine has been shown to proceed directly between the two

substrates with inversion of configuration at phosphorous and without covalent interference of the enzyme, typical of an in-line S_N2 displacement [36]. By means of site directed mutagenesis, we have replaced D316 with either an asparagine or a glutamic acid residue. Both of these changes lead to nearly complete loss of AK activity (activity in both cases reduced by more than 5 orders of magnitude). The loss of activity on replacement of aspartic acid at D316 with asparagine is expected, as it should not be able to serve as the proton acceptor. Computational analysis of the active site mutant D316E suggests that 3 of 8 possible rotamers may position OD2 glutamic acid within 3 Å of the 5'-hydroxyl of adenosine. However, all these rotamers show steric hindrance with other residues within the active site pocket, accounting for the loss of activity. Thus, the observed effect of the amino acid replacements at position D316 are consistent with the proposal that this group acts as a catalytic base in the reaction mechanism of AK.

3.9 References

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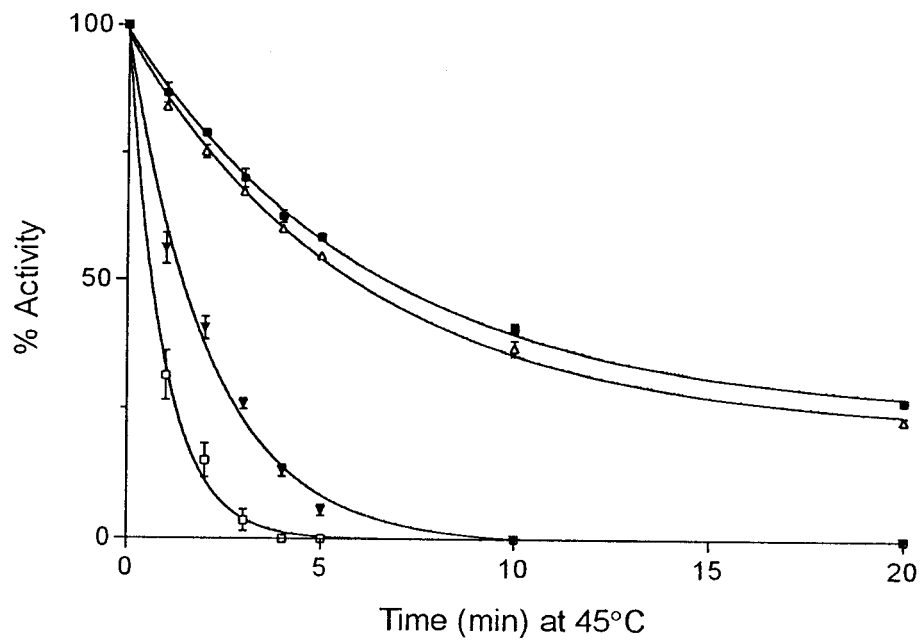
3.10 Appendix

Figure 3.7 Thermal inactivation kinetics

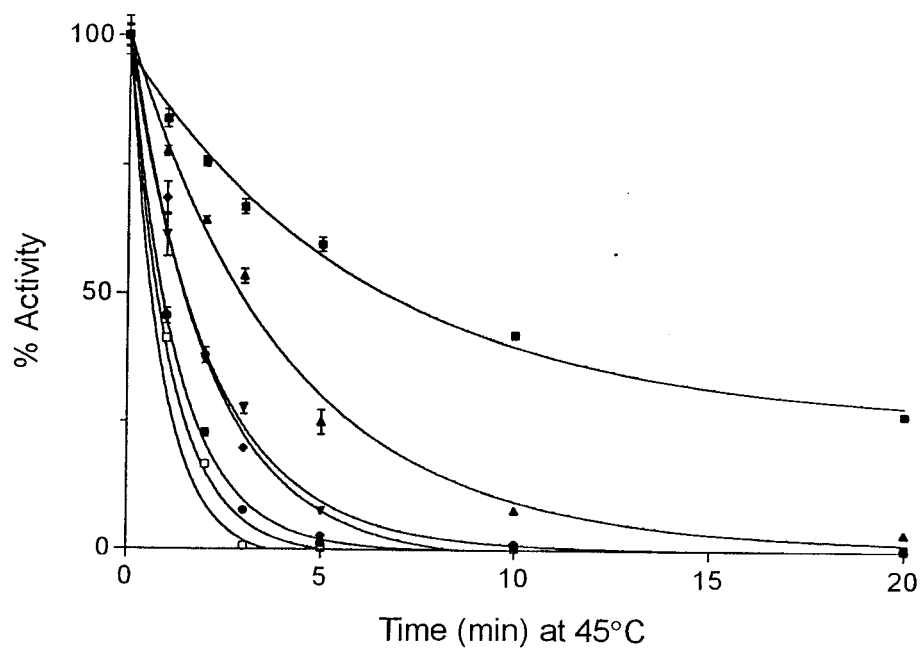
Cell extracts were incubated at 45°C, and the percent activity was plotted against the time of incubation. A) N-terminal mutants were incubated at 45°C and initial velocity was determined for: (■) wild-type; (△) N1; (▼) N4; (□) N5. B) C-terminal mutants were incubated at 45°C and the initial velocity was determined for: (■) wild-type; (▲) C1; (▼) C2; (◆) C3; (●) C4; and (□) C5. The reaction mixture contained 10μM adenosine, 1 mM ATP, 2.46 mM MgCl₂ and 100 mM phosphate buffer pH 7.4 at 37°C.

Fig. 3.7

A



B



Chapter 4. The Effect of Inorganic Phosphate on the Activity of Bacterial Ribokinase

Mary C. Maj and Radhey S. Gupta (2001) *Journal of Protein Chemistry*, 20, (2), 139-144.

4.1 Preface

In chapter 3, the contribution of residues at the N- and C-terminal regions to the activity and thermal stability as well as mutation of the putative catalytic base were examined. The putative catalytic aspartic acid residue is absolutely conserved among adenosine kinases. This aspartic acid residue is also structurally conserved at the active site of bacterial ribokinase. Both adenosine kinase and ribokinase belong to the PfkB family of carbohydrate kinase, named after the group of enzymes which bear two sequence motifs found in the minor form of microbial phosphofructokinase. Three dimensional structures of both adenosine kinase and bacterial ribokinase have been solved, revealing remarkable similarity though the amino acid sequence conservation is less than 30%. It has been suggested that PfkB kinases will also share similar catalytic mechanisms, though this issue has not been previously examined.

Initial activity studies of ribokinase employed the indirect assay of the pyruvate kinase/lactate dehydrogenase system which couples the production of ADP by ribokinase to the conversion of NAD from NADH through the dephosphorylation of phosphoenolpyruvate. This system was found to have low sensitivity and reproducibility. A stopped radioactive assay, similar to that used for the study of adenosine kinase activity was developed.

The results of this chapter reveal that the activity of bacterial ribokinase shares with adenosine kinase both pentavalent ion stimulation and decrease in K_m of the

phosphate accepting co-substrate. Surprisingly, it was found that phosphoenol pyruvate could also stimulate activity and decrease the K_m for ribose, which may have contributed to the low reproducibility of the coupled assay found by us and by other groups.

I performed all studies presented in this chapter. The purified bacterial ribokinase was supplied by Mark A. Hermodson and Jelena Ziatseva of Purdue University. Table 4.1 has been altered from the published paper to include the first order rate constant k_{cat} and the second order rate constant k_{cat}/K_m . A ribbon diagram, based on crystallographic data, showing the structural similarity between ribokinase and adenosine has been included as Figure 4.4 found in section 4.11, Appendix A.

4.2 Paper

The Effect Of Inorganic Phosphate On The Activity Of Bacterial Ribokinase

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4.3 Abbreviations

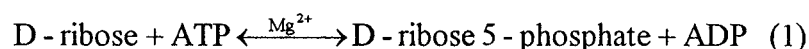
RK, Ribokinase; AK, adenosine kinase; Pi, inorganic phosphate; PVI, pentavalent ions;
R-5-P, D-ribose 5-phosphate; PEP, phosphoenolpyruvate; PfkB, phosphofructokinase B.

4.4 Abstract

Ribokinase and adenosine kinase are both members of the PfkB family of carbohydrate kinases. The activity of mammalian adenosine kinase was previously shown to be affected by pentavalent ions (PVI). We now present evidence that the catalytic activity of *E. coli* ribokinase is also affected by PVI, increasing both the velocity and affinity of the enzyme for D-ribose. The K_m for ribose decreased from 0.61 mM to 0.21 mM, 0.22 mM and 0.33 mM in the presence of 20 mM phosphate, arsenate and vanadate respectively. The activity of ribokinase was stimulated in a hyperbolic fashion, maximum velocity increasing 23-fold, 13-fold, and 11-fold under the same conditions, respectively. Activity was also affected upon by the addition of phosphoenolpyruvate, suggesting that phosphorylated metabolites could be involved enzymatic control. The similar effect of PVI on distantly related enzymes suggest that a common mechanism for activity is shared among PfkB family members.

4.5 Introduction

Ribokinase (RK, EC 2.7.1.15) or ATP:D-ribose 5-phosphotransferase is a carbohydrate kinase which catalyses the phosphorylation of ribose to ribose 5-phosphate (R-5-P) according to the reaction:



R-5-P is then retained by the cell and further metabolized to nucleotides. R-5-P is also an essential intermediate for the synthesis of phosphoribosyl pyrophosphate, which is required for numerous biosynthetic reactions.

Ribokinase (RK) belongs to the PfkB family of carbohydrate kinases [1,2]. Members of this family include adenosine kinase (EC 2.7.1.20), inosine-guanosine kinase (EC 2.7.1.73), fructokinase (EC 2.7.1.4), 1-phosphofructokinase (EC 2.7.1.56), 2-dehydro-3-deoxyglucokinase (EC 2.7.1.45) and 6-phosphofructokinase minor (EC 2.7.1.11). The overall sequence identity between family members is less than 30%. The three-dimensional structures of two family members have recently been solved. High structural similarity is seen between RK [3] and adenosine kinase (AK) [4;5]. Both proteins are comprised of a large $\alpha\beta\alpha$ sandwich domain and a smaller domain that acts as a lid over the active site (Fig. 4.4, Appendix A).

The members of the PfkB family are identified by two highly conserved sequence motifs. The first motif is found near the N-terminus. Crystallographic data for bacterial

ribokinase [6] and AK from *Toxoplasma gondii* [5] suggest that this motif contains two structurally significant consecutive glycine residues. Upon substrate binding, this 'GG switch' region undergoes torsional changes, which causes a rigid body rotation of the large and small domains. This rotation serves to create the co-substrate ATP binding site. The second motif is found near the C-terminus. Structural evidence suggests these residues are involved in ATP binding and the formation of an anion hole [5;6]. This sequence motif also contains an absolutely conserved aspartic acid residue which serves as the catalytic base [3-7]. These structural data as well as biochemical evidence for AK [8] support the notion that phosphate transfer occurs by an in-line mechanism.

Strong sequence identity within the two motifs, the absolute conservation of a catalytic base, and the remarkable structural similarity between RK and AK suggest that PfkB carbohydrate kinases will also share similar catalytic mechanisms [4]. We have recently shown that AK activity is almost completely dependant upon inorganic phosphate and other pentavalent ions (PVI) at acidic pH values [9] which mimic conditions of ischemia [10;11]. The presence of phosphate serves to decrease the K_m for adenosine as well as protect the enzyme from substrate inhibition at both acidic and physiologic pH [9]. To explore the relationship of PfkB proteins, we have investigated the activity of RK in the presence of phosphate, arsenate, vanadate and phosphoenolpyruvate. Our data show that the activity of RK, similar to AK, is affected by PVI. An improved assay for the detection of D-ribose phosphorylation (R-5-P) activity is also reported.

4.6 Methods and Materials

4.6.1 Materials

D-[1-³H] ribose (20Ci/mmol) was obtained from American Radiolabelled Chemicals Inc., St. Louis, MO. A stock solution of D-[1-³H] ribose to be used in the radioactive assay was made by diluting the original stock with unlabeled D-ribose to give a specific activity of 100 mCi/mmol. ATP was purchased from Pharmacia Biotech (Canada). D-ribose was a product of J.T. Baker Chemical Co. (Phillipsburg, NJ). All other chemicals were of analytical reagent grade.

Purified recombinant RK, a product of the *RbsK* gene of *E. coli* [12;13], was generously supplied by Dr. Mark A. Hermodson from the Department of Biochemistry at Purdue University, West Lafayette, Indiana. The concentrated purified protein was stored in 10mM Tris-HCl, pH 8.0, 10 mM MgCl₂, 1mM DTT and 50% glycerol at -20°C.

4.6.2 Activity Assay

RK activity was assayed by a stopped radiochemical method using D-[1-³H] ribose, similar to the assay for AK [9;14;15]. Assays were performed at 37°C in 100 mM Tris-maleate buffer, 125 mM potassium chloride and 0.04 µg of the purified protein. The pH of all reagents was adjusted to that of the assay. For the *K_m* determination of ribose, the concentration of ATP was held fixed at 5 mM, whereas the *K_m* determination of ATP was carried out in the presence of 2 mM ribose. In all cases, the concentration of free magnesium was fixed at 2.5 mM. The reaction was initiated upon the addition of RK. At various time

intervals within the linear reaction range, 50 μL aliquots were withdrawn and immediately placed in ice-cold lanthanum chloride to precipitate the phosphorylated product of the RK assay. The precipitate was collected after a minimum of 3 h by suction filtration on a glass fiber filter, washed with 20 volumes of cold water, dried, and then placed in scintillation vials with 5 mls aqueous scintillation fluid. The radioactivity was counted on a Beckman LS 7800 scintillator.

The amount of radioactive product (R-5-P) was plotted against time. The slope of the line was used to determine the initial velocity. The initial velocities were then plotted as a function of substrate concentration to determine K_m values for both ribose and ATP. All data were computed with Graphpad Prism software.

4.7 Results

4.7.1 Development of the Stopped Radioactive Assay

The activity of RK is generally measured by a coupled assay [12] that uses the product of the RK reaction, ADP, as co-substrate with phosphoenolpyruvate for the enzyme pyruvate kinase. Pyruvate, produced in the latter reaction, is reduced by NADH in the presence of lactate dehydrogenase. The oxidation of NADH is followed spectrophotometrically. When we initially examined the effect of P_i , this assay was found to have low sensitivity and reproducibility. Therefore, we proceeded to develop a radioactive assay for RK that directly measures the production of ^3H -Ribose-5- PO_4 . The radioactive assay, described in section 4.6.2, was very sensitive and highly reproducible.

4.7.2 Effects of Pentavalent ions on Activity and K_m D-ribose

The catalytic activity of RK is almost completely dependant upon the presence of PVI at pH 6.2 (Figure 4.1 A-C). The velocity of RK increases hyperbolically with increasing ribose concentration from 0.01 mM to 1 mM. No substrate inhibition was seen with concentrations of ribose up to 20 mM. The addition of PVI serves to increase the maximum velocity of RK as well as decrease the K_m for ribose. The effect of phosphate, arsenate and vanadate are summarized in Table 4.1. The K_m for ribose decreases from 0.65 mM to 0.21 mM, 0.22 mM and 0.33 mM with 20 mM phosphate, arsenate and vanadate respectively. At pH 7.4, activity is increased 5-fold and K_m is reduced 40% in the presence of 20 mM phosphate (Table 4.1).

The pentavalent ion dependency of RK activity at pH 6.2 is shown in Figure 4.2. The maximum velocity of RK increases hyperbolically with increasing PVI concentrations. The V_{max} in the absence of PVI is 7.6 pmoles R-5-P formed/min. At saturating phosphate, arsenate and vanadate, V_{max} is increased to 193, 101 and 82 pmoles R-5-P formed/min respectively. No inhibition of activity was seen up to 100 mM PVI. The concentration of PVI at half maximal velocity is 2.2 mM for phosphate, 3.8 mM for arsenate and 5.6 mM for vanadate. Phosphate, arsenate and vanadate are all moderately basic anions with four oxo-groups. The ionic radii of the central atom with the oxidation number of 5+ is 52 pm for phosphorus, 60 pm for arsenic and 68 pm for vanadium. Therefore, the efficacy of PVI to stimulate activity may be correlated to its steric ability to bind the protein.

Figure 4.1. The Effect of Pentavalent Ions on K_m D-ribose.

The initial velocity is plotted against increasing concentrations of D-ribose. (A)

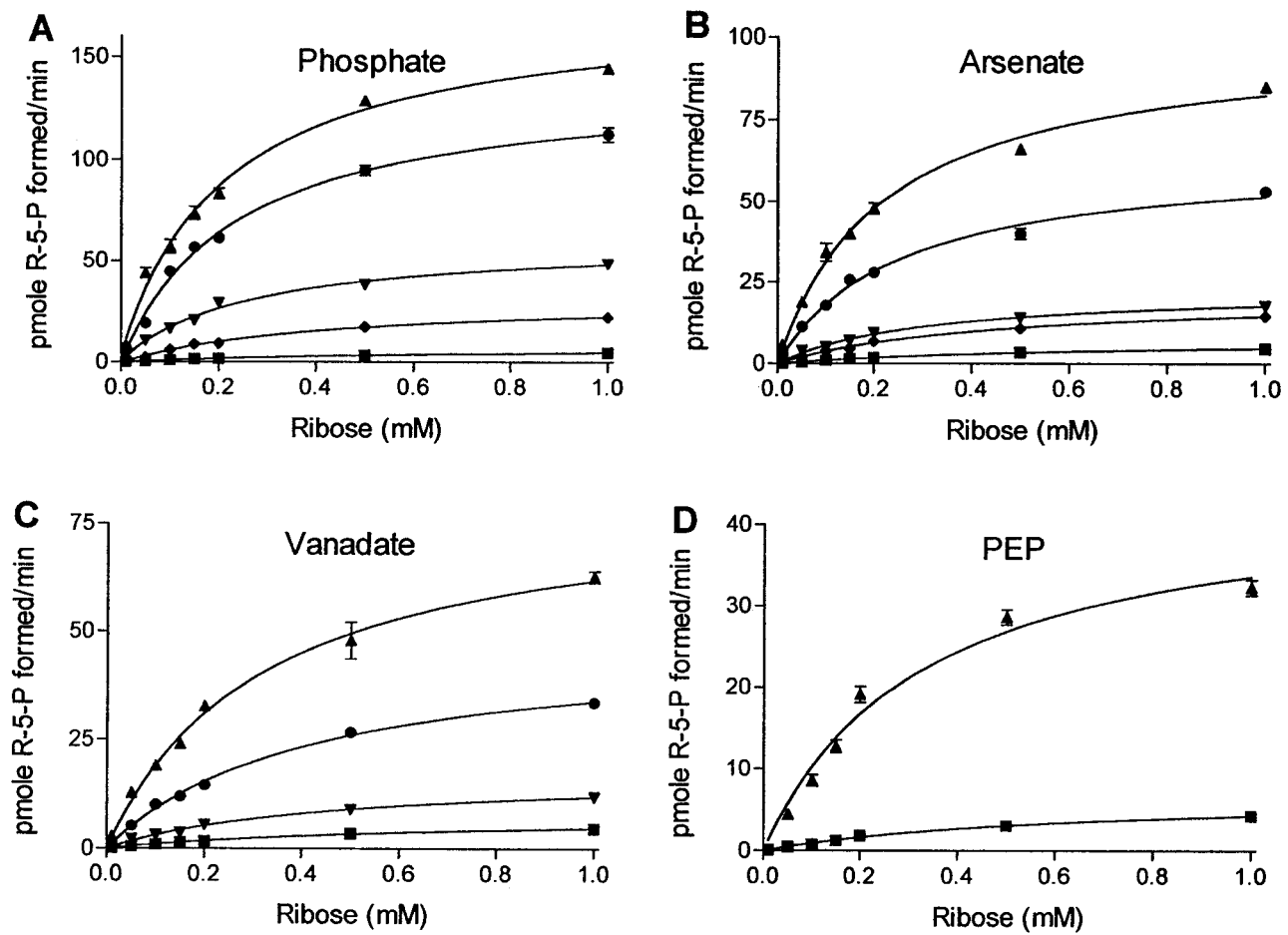
Phosphate: 0 (■); 0.5 mM (◆); 1 mM (▼); 5 mM (●) and 20 mM (▲). (B) Arsenate: 0

(■); 0.5 mM (◆); 1 mM (▼); 5 mM (●) and 20 mM (▲). (C) Vanadate: 0 (■); 1 mM (▼);

5 mM (●) and 20 mM (▲). (D) Activity in the absence (■) and presence (▲) of 20 mM

phosphoenol pyruvate. The error bars shown in all figures are derived from the standard error of the initial velocity, and they do not represent results from replicate experiments.

Fig. 4.1



4.7.3 Effects of Phosphoenolpyruvate on Activity and Affinity for D-ribose

Our inability to see any significant effect of Pi on RK activity using the coupled assay prompted us to examine whether phosphoenolpyruvate (PEP), which is present in the coupled assay, had any effect on RK activity. The effect of PEP was examined using the radioactive assay. Results of these studies (Figure 4.1 D) show that the addition of 20 mM PEP served to increase the maximum velocity almost 7-fold, and to decrease the K_m of ribose from $650 \pm 60 \mu\text{M}$ to $335 \pm 70 \mu\text{M}$.

4.7.4 Effects of Pentavalent Ions on Activity and Substrate Affinity for ATP

Initial activity studies had shown that magnesium is required for activity. However, RK, similar to AK, exhibits inhibition at high concentrations of free Mg^{2+} . The optimum concentration of free Mg^{2+} at pH 6.2 was determined to be 2.5 mM. Concentrations of free Mg^{2+} above 2.5 mM showed gradual inhibition of activity. RK exhibits typical Michaelis-Menten kinetics with increasing concentrations of ATP up to 5 mM. ATP concentrations above 5 mM appeared inhibitory.

The addition of PVI has little effect on the K_m of RK for ATP (Figure 4.3) changing from 2.6 mM in the absence of any PVI to 2.5 mM in the presence of 20 mM phosphate, arsenate and vanadate. However, the maximum velocity was increased from 8.1 ± 0.4 pmoles R-5-P formed/min to 177 ± 7.1 , 102 ± 4.2 and 90 ± 5.8 pmoles R-5-P formed/min upon addition of 20 mM phosphate, arsenate and vanadate respectively.

Table 4.1 Effect of Pentavalent Ions on the Activity and K_m of Ribokinase for D-ribose

Pentavalent ion	Pentavalent ion (mM)	K_m ribose (mM)	V_{max} (pmolesR-5-P formed/min)	k_{cat} (s ⁻¹)	k_{cat}/K_m M ⁻¹ s ⁻¹
Phosphate pH 6.2	0	0.65 ± 0.09	7.6 ± 0.4	0.61 ± 0.03	9.43 × 10 ²
	0.5	0.43 ± 0.08	32.0 ± 2.1	2.58 ± 0.17	6.00 × 10 ³
	1	0.27 ± 0.06	59.6 ± 3.1	4.81 ± 0.25	1.78 × 10 ⁴
	5	0.23 ± 0.04	139 ± 6.2	11.3 ± 0.50	4.89 × 10 ⁴
	20	0.21 ± 0.04	172 ± 8.4	13.9 ± 0.68	6.62 × 10 ⁴
Phosphate pH 7.4	0	0.18 ± 0.01	193 ± 7.0	15.6 ± 0.65	8.64 × 10 ⁴
	20	0.11 ± 0.02	1100 ± 99	88.8 ± 7.9	8.07 × 10 ⁵
Arsenate pH 6.2	0.5	0.48 ± 0.05	21.3 ± 1.1	1.72 ± 0.09	3.58 × 10 ³
	1	0.32 ± 0.04	23.2 ± 1.1	1.87 ± 0.09	5.85 × 10 ³
	5	0.26 ± 0.03	64.5 ± 3.2	5.20 ± 0.26	2.00 × 10 ⁴
	20	0.22 ± 0.02	101 ± 4.2	8.11 ± 0.34	3.69 × 10 ⁴
Vanadate pH 6.2	1	0.46 ± 0.07	17.4 ± 1.2	1.40 ± 0.10	3.05 × 10 ³
	5	0.43 ± 0.04	48.1 ± 2.1	3.88 ± 0.17	9.02 × 10 ³
	20	0.33 ± 0.03	81.8 ± 3.5	6.60 ± 0.28	2.00 × 10 ⁴

Figure 4.2 Effect of Pentavalent Ions on the Maximum Velocity.

V_{max} derived from Figure 4.1 is plotted vs. pentavalent ions: (■) phosphate; (▲) arsenate; and (▼) vanadate.

Fig. 4.2

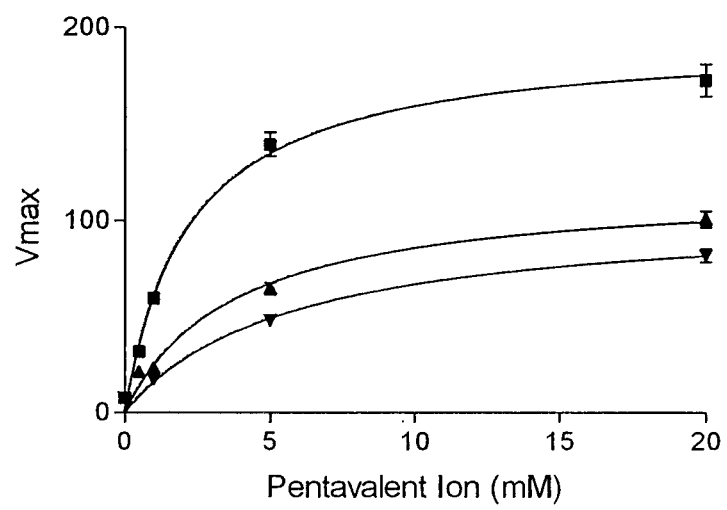
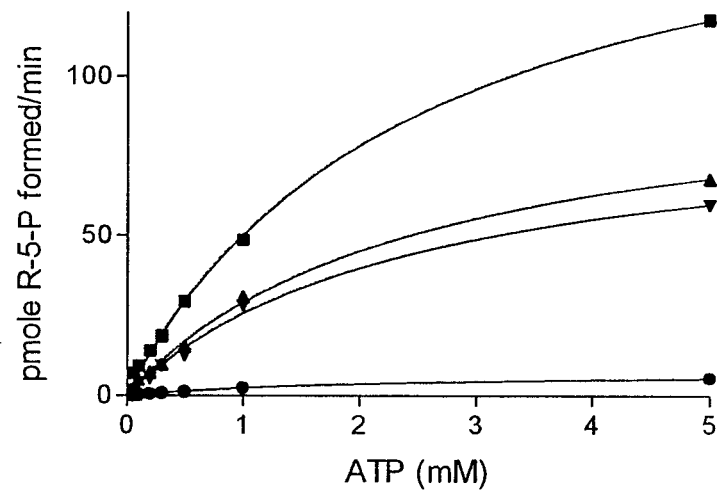


Figure 4.3 The Effect of Pentavalent Ions on K_m ATP.

Initial velocity is plotted vs. ATP concentration: in the absence of ions (●); 20 mM phosphate (■); 20 mM arsenate (▲); and 20 mM vanadate (▼).

Fig. 4.3



4.8 Discussion

Despite low sequence identity between AK and RK, their overall three-dimensional structures are surprisingly similar. Based on the structural similarity and homology of residues at the active site, as well as two highly conserved sequence motifs that identify the family of PfkB kinases, it is thought that all family members of this class of enzymes would have similar catalytic mechanisms [5]. The focus of this study was to determine if RK activity, like AK, is affected by the presence of inorganic phosphate and other PVI. The addition of phosphate to the reaction of AK serves to stimulate activity by both decreasing the *K_m* for adenosine and relieving inhibition at high concentrations of adenosine [9]. Other cations and anions (K^+ , Na^+ , Fe^{2+} , Cu^{2+} , Ca^{2+} , Zn^{2+} , acetate, sulfate, sulfite, nitrate, nitrite and carbonate) were found to have no effect on AK activity [15]. We have previously shown that phosphate is not consumed in the catalytic reaction of AK nor is a phosphoryl enzyme intermediate formed [15;16]. The data presented here shows that the presence of PVI serves to increase maximum velocity of RK as well as decrease the *K_m* for D-ribose. The ability of arsenate and vanadate to substitute for phosphate suggests that phosphate is not consumed in the catalytic reaction of RK. The effect of PVI on these two distantly related kinases suggest that this may be an intrinsic property of the PfkB family of proteins.

In addition to RK an AK, three other enzymes that are involved in the transfer of phosphate from ATP to a sugar derivative have been reported to show similar dependence on phosphate for activity. Phosphate is thought to invoke an active configuration for both the bifunctional 6-phosphofructo-2-kinase (EC 2.7.1.105) [17] and phosphoribosylpyrophosphate synthetase (EC 2.7.6.1) [18]. Phosphate has also been found to stimulate the activity of 6-phosphofructokinase (EC 2.7.1.11) [19] and relieve inhibition at high concentrations of ATP [20] though it is not clear which isoforms are discussed. The phosphate binding site has not been determined for any of these proteins.

RK has recently been crystallized in the presence of 2 M $\text{NH}_4\text{H}_2\text{PO}_4$ as the precipitant [3]. The structure reveals a phosphate group bound near the active site. The phosphate group interacts with residues Glu190 and Asn187. The O3 atom of this phosphate is within 5 Å of the O2B atom of ADP. The position of this phosphate group appears far enough from the active site as not to represent the γ -phosphate of ATP yet may be close enough to the substrate binding site to affect activity. Interestingly, a glutamate and asparagine residue occupies similar three dimensional space at the active site of AK [4].

Our survey of the literature has revealed a number of similarities among PfkB family members. AK (EC 2.7.1.20) [21;22] and fructokinase (EC 2.7.1.4) [23], are both inhibited by excess free magnesium and excess free ATP. An optimum Mg/ATP ratio is required for the activity of 1-phosphofructokinase (EC 2.7.1.56) [24], 6-phosphofructokinase minor (EC 2.7.1.11) [27] and inosine/guanosine kinase (EC 2.7.1.73) [26]. Product inhibition studies of inosine/guanosine kinase [26],

6-phosphofructokinase minor [25] and AK [28] as well as structural studies of AK [4;5] and RK [3;6] support an ordered Bi Bi mechanism in which ATP is the last substrate to bind and ADP the first product to be released.

Structural information for RK and AK show evidence that conformational changes upon substrate binding are an integral part of their kinetic mechanism [6]. Conserved residues at the active sites further suggest similar mechanisms for 5'-carbohydrate phosphorylation. The observations that phosphate stimulates activity and increases the enzyme affinity for the first substrate to bind provide additional evidence that a common mechanism likely to applies to related carbohydrate kinases. The precise role that PVI play in enhancing the catalytic activity of RK or AK is presently not clear. It is possible that PVI exert their effects by stabilizing a certain 'open' configuration of the protein. This would allow the first substrate to bind with a reduced K_m as well as reduce the non-specific binding of co-substrate Mg/ATP^{2-} . Phosphate may also facilitate the reorganization of protons to regenerate the active protein upon enzyme turnover.

4.9 Acknowledgments

We gratefully thank Mark A. Hermodson and Jelena Ziatseva from the Department of Biochemistry, Purdue University, West Lafayette, Indiana, for supplying us with purified ribokinase. This work was supported by a research grant to R.S.G. and by a research traineeship award to M.C.M. from the Heart and Stroke Foundation of Canada.

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4.11 Appendix A

Figure 4.4 Ribbon Diagram of RK and AK.

The tertiary structure of one bacterial ribokinase subunit is compared to the structure of the monomeric adenosine kinase enzyme. Both proteins are comprised of a large $\alpha\beta\alpha$ domain and a smaller domain which acts as a lid over the active site. The absence of the α -helices on the outside face of the ribokinase lid domain allows for the formation of the ribokinase dimer.

Fig. 4.4



Chapter 5. Pentavalent Ion Dependency is a Conserved Property of Adenosine Kinase from Divergent Sources: Identification of a Novel Motif Implicated in Phosphate and Magnesium Ion Binding and Substrate Inhibition.

Mary C. Maj, Bhag Singh and Radhey S. Gupta (2002) *Biochemistry*, 41 (12): 4059-69.

5.1 Preface

The effects of PVI on the activity of mammalian adenosine kinase and bacterial ribokinase were studied in Chapters 2 and 4 respectively. To further examine the role of pentavalent ions on the activity of adenosine kinase, this paper investigated the effects of PVI on AK from a variety of sources including plant, yeast and parasitic organisms. AK from the parasitic organism *L. donovani* has not been shown to be inhibited by high concentrations of adenosine and magnesium. The presence of PVI served to stimulate activity and decrease the K_m for adenosine for all enzymes studied.

An amino acid motif, NXXE, is absolutely conserved for AK and RK, and is highly conserved among PfkB family members. The 3D structure of RK shows a bound phosphate ion at the NXXE motif. As the activity both RK and AK from all sources studied are stimulated by the addition of phosphate, N239 and E242 of the Chinese hamster enzyme were mutated, and phosphate effects investigated. The mutation of N239 resulted in a protein which was not stimulated by phosphate nor exhibited inhibition by high concentrations of substrate and free magnesium. Mutation of E242 resulted in a protein with greatly altered phosphate stimulation and magnesium inhibition.

The results of these studies suggest that the conserved residues of the NXXE motif are involved in the regulation of activity. Inhibitors of the enzyme adenosine kinase are currently being investigated for the therapeutic treatment of a number of disease processes. As this motif is found less than 10Å from the putative catalytic base,

inhibitors of AK which are extended to bind at the conserved site may result in increased specificity of binding and decrease the likelihood of being displaced by natural substrates.

I performed all experimental work presented in this paper with the exception performing the PCR reaction to generate the site-directed mutants, which were carried out by Dr. B. Singh. Grateful acknowledgements are extended to Doctors Weretilnyk and Summers of McMaster University for supplying cell extracts from spinach, and Dr. Reiner from the University of British Columbia for *L. donovani* cells.

Secondary plots from which the data found in Tables 5.1 and 5.2 are derived are included in section 5.11, Appendix.

5.2 Paper

Pentavalent Ions Dependency is a Conserved Property of Adenosine Kinase from Diverse Sources: Identification of a Novel Motif Implicated In Phosphate and Magnesium Ion Binding and Substrate Inhibition †

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5.3 Abbreviations:

adenosine, ado; adenosine kinase, AK; arsenate, AsPO₄; Chinese hamster ovary, CHO; free magnesium, Mg²⁺; magnesium coordinated to 6 water molecules, MO₆; pentavalent ions, PVI; phosphate, PO₄; ribokinase, RK; vanadate, VO₄.

5.4 Abstract

The catalytic activity of adenosine kinase (AK) from mammalian sources has previously been shown to exhibit a marked dependency upon the presence of pentavalent ions (PVI) such as phosphate (PO_4), arsenate or vanadate. We now show that the activity of AK from diverse sources, including a plant, yeast and protist species, is also markedly enhanced in the presence of PVI. In all cases, PO_4 , or other PVI exerted their effects primarily by decreasing the K_m for adenosine and alleviating the inhibition caused by high concentrations substrates. These results provide evidence that PVI dependency is a conserved property of AK and perhaps of PfkB family of carbohydrate kinases which includes AK. Based on sequence alignments, we have identified a conserved motif NXXE within the PfkB family. The N and E of this motif make close contacts with Mg^{2+} and PO_4 ions in the crystal structures of AK and bacterial ribokinase (another PfkB member which shows PVI dependency), implicating the residues in their binding. Site directed mutagenesis of these residues in Chinese hamster AK have resulted in active proteins with greatly altered phosphate stimulation and substrate inhibition characteristics. The N239Q mutation leads to the formation of an active protein whose activity was not stimulated by PO_4 or inhibited by high concentrations of adenosine or ATP. The activity of the E242D mutant protein was also not significantly altered in the presence of phosphate. Although PO_4 had no effect on the $K_m^{\text{Adenosine}}$ for this mutant, the K_m^{ATP} , $K_i^{\text{Adenosine}}$ and K_i^{ATP} were significantly decreased. In contrast to these mutations,

N239L or E242L mutant proteins showed greatly decreased activity with altered Mg^{2+} requirement. These observations support the view that N239 and E242 play an important role in the binding of PO_4 and Mg^{2+} ions required for the catalytic activity of adenosine kinase.

5.5 Introduction

The enzyme adenosine kinase (AK)¹ is a purine salvage enzyme which catalyses the phosphorylation of the 5' hydroxyl of adenosine via the γ -phosphate of co-substrate Mg/ATP²⁻. AK is widely distributed among plants, eukaryotic micro-organisms and mammalian tissues [1-25]. An important regulatory feature of AK is its potent inhibition by high concentrations of adenosine. Studies have shown that during myocardial and cerebral ischemia, there are marked increases in the local concentration of adenosine [26;27] correlated with a decrease in pH to as low as 6.0 [28-31]. Elevated levels of adenosine has been associated with both cardiac [26;32] and neural protection [27;33], and AK is indicated to be the primary enzyme involved in its regulation under both physiological and ischemic conditions [7;31;31].

The mechanism of AK activity reportedly varies among enzyme sources, though the majority of studies on mammalian enzymes suggest a sequential mechanism [34-38]. However, these studies report differing orders of substrate binding and product release. Difficulties in the interpretation of AK kinetic data giving rise to conflicting results is in part due to inhibition of AK activity by high concentrations of adenosine, excess free magnesium (Mg²⁺) and excess ATP⁴⁻. Widely different assay conditions have been reported, revealing an intricate relationship between pH and initial velocity. It is well documented that the optimum pH for activity is dependant on the ratio of ATP/Mg²⁺

[10;34;39-41]. Optimum activity is also dependant on the ratio of Mg^{2+} /adenosine [10;41;42]. A depression in pH, as that which occurs during ischemia thus can have a marked effect on the activity of AK.

AK belongs to the PfkB family of carbohydrate kinases, classified on the basis of two unique sequence motifs. Members of this family include ribokinase (RK:EC 2.7.1.15), inosine-guanosine kinase (EC 2.7.1.73), fructokinase (EC 2.7.1.4) and 1-phosphofructokinase (EC 2.7.1.56) [43;44]. The 3D structure of two PfkB family members, AK and RK, have been solved and are found to be remarkably similar [45-48]. The structure of bacterial RK was resolved with ribose, ADP, and a bound phosphate near the active site [47]. The distance of the phosphate ion from the β -phosphate of ADP suggests that this phosphate does not represent the γ -phosphate of ATP. This phosphate ion makes close contacts with conserved asparagine and glutamic acid residues at sequence positions 187 and 190 respectively (Figure 5.1 B). The corresponding residues (positions 223 and 226 in the human sequence and positions 239 and 242 in Chinese hamster sequence), are conserved in AK and occupy similar 3D space (Figure 5.1 A). The Asn and Glu residues at these positions are also conserved in other proteins belonging to the PfkB family of carbohydrate kinases thus identifying a new sequence motif NXXE, which is a common characteristic of these proteins (Figure 5.2).

The human AK protein was crystallized in the presence of adenosine and magnesium. The three-dimensional structure shows two adenosine molecules and a magnesium ion, which has octahedral coordination to six ordered waters (MO_6), bound at the active site [45]. Five of these six ordered waters make H-bond interactions with the

protein, which include side chain contacts with conserved NXXE residues asparagine 223 and glutamic acid 226 as well as with the putative catalytic base, aspartic acid 300 (Figure 5.1 A). MO_6 interacts with $\text{O5}'$ of adenosine by a close contact of 3.52\AA as well as 2 H-bonds through a bridging water molecule. The position of MO_6 differs from Mg^{2+} found in the active site of *Toxoplasma gondii* AK (Figure 5.1 C), which is coordinated between the α and β phosphates of AMP-PCP (a non-hydrolysable ATP analogue), though forms only one hydrogen bond with a non-bridging oxygen of the β -phosphate [46]. This Mg^{2+} does not show typical octahedral coordination as shown for MO_6 . For clarity, we have designated Mg^{2+} which is coordinated to AMP-PCP as M1, and the magnesium species MO_6 which is located near the $5'$ -hydroxyl of adenosine as M2.

We have shown that the activity of mammalian AK as well as bacterial RK is greatly affected by pentavalent ions (PVI) such as phosphate, arsenate and vanadate. The maximum velocity increased and K_m for the phosphate accepting substrate were decreased in the presence of PVI at physiological pH [49;50-52]. At more acidic pH values, which mimic conditions of ischemia [49;50], AK activity showed an absolute requirement for the presence of PVI. To determine if this effect is conserved among species, we have examined the effect of PVI on the activity of AK from Chinese hamster ovary (CHO) cells, spinach, *Saccharomyces cerevisiae* and *Leishmania donovani*. Our results show that the PVI dependency, or stimulation of AK activity by these ions, is a conserved property of AK from various sources.

Figure 5.1 The Active-Site Structure of Adenosine Kinase and Ribokinase.

The hydrogen-bond network between residues and active site ligands are shown. The putative catalytic base, aspartic acid, and NXXE residues asparagine and glutamic acid are absolutely conserved among AK and RK. (A) Structure of human AK active site, with the corresponding residue numbering for CHO AK. H-bonding from M2 to N239 and E242 are shown. The approximate position of R148 in its resting state is also depicted. (B) Ribokinase active site is shown with ligands ribose and ADP. The relative position of PO₄ and interactions with asparagine and glutamic acid are shown. (C) Structure of *T. gondii* AK. An arginine residue which is absolutely conserved among AK, undergoes domain and side-chain rotation to position near the γ -phosphate of AMP-PCP. M1 is coordinated between the α - and β -phosphates of AMP-PCP.

Figure 5.1.

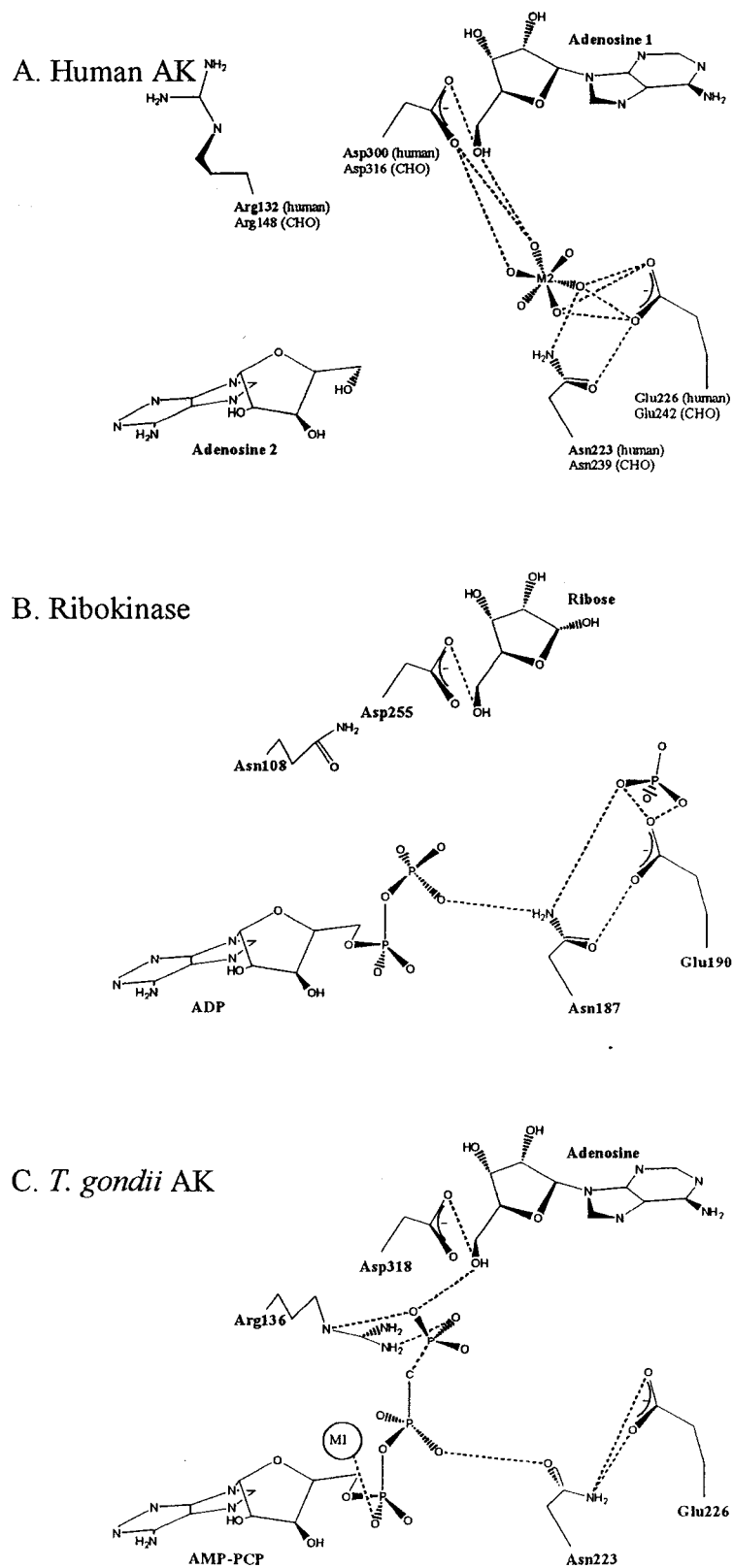


Figure 5.2 Sequence Alignment of Representative Proteins from the PfkB Family of Carbohydrate Kinases.

The conserved NXXE motif is marked with asterisks and highlighted in bold type. The sequence identifications are as follows: AK-CHO, Chinese hamster ovary AK; AK-HUM, human AK; AK-SC, *S. cerevisiae* AK; AK-LD, *L. donovani* AK; AK-TG, *T. gondii* AK; AK-AT, *A. thaliana* AK; RK-EC, *E. coli* ribokinase; PK-HUM, human pyridoxal kinase; TH-BS, *B. subtilis* phosphomethylpyrimidine kinase; T6PK-SA, *S. aureus* tagatose-6-phosphate kinase; K1PF-EC, *E. coli* 1-phosphofructokinase; K6P2-EC, *E. coli* 6-phosphofructokinase isozyme 2; AHS-EC, *E. coli* ADP heptose synthase; IGK-EC, *E. coli* inosine/guanosine kinase.

Figure 5.2.

* *

AK-CHO	PYVDILFGNETEAATFAREQ
AK-HUM	PYVDILFGNETEAATFAREQ
AK-SC	PYATVIANESEAEAFCDAF
AK-LD	PYLDVIFGNEVEAKALADAM
AK-TG	LHTNILFGNEEEFAHLAKVH
AK-AT	PYMDFVFGNETEARTFSRVH
RK-EC	ALVDIITPNETEAEKLTGIR
PK-HUM	PLADIITPNQFEAELLSGRK
TH-BS	PLALVITPNLFEASQLSGMD
T6PK-SA	YKPTVIKPNISELYQLLNPL
K1PF-EC	AAPWLVKPNRRELEIWAGRK
K6P2-EC	GNIELVKPNQKELSALVNRE
AHS-EC	RGATLLTPNLSEFEAVGKC
IGK-EC	DHVSILAMNEDEAEALTGES

The catalytic activities of both AK as well as bacterial RK are also inhibited by high concentrations of Mg^{2+} . Since both PO_4 and Mg^{2+} interact with the asparagine and glutamic acid residues of the NXXE motif, the possibility that this motif may be involved in their binding and catalytic effect was studied. Our studies show that the mutational alterations of Asn239 and Glu242 in CHO AK sequence resulted in proteins with greatly altered PO_4 effect and increased tolerance for Mg^{2+} , providing evidence that these residues are involved in PO_4 and Mg^{2+} binding and their effects on AK activity.

5.6 Methods and Materials

5.6.1 Cell Extracts

Wild-type CHO cells grown in alpha-MEM media supplemented with 5% fetal bovine serum were harvested near confluency, pelleted and washed [53]. The cell pellet was suspended in 50 mM Tris-maleate buffer pH 7.4 and 100 mM NaCl, sonicated to break open the cells and then centrifuged to remove cellular debris. The cell lysate was stored at $-70^{\circ}C$ after supplementing it with 5% glycerol, 0.1 mM DTT and EDTA (final concentrations). Yeast DL-1 *Saccharomyces cerevisiae* was grown in YPD media supplemented with 2% dextrose for 48 hours. The pellet from a 10 mL culture was re-suspended in 50 mM Tris-maleate buffer pH 7.4 containing 100 mM NaCl. The suspension was vortexed with 425-600 micron glass beads for 6 x 30 second bursts, then centrifuged to remove cellular debris. The cell extract was stored at $-70^{\circ}C$ after addition of 5% glycerol and 0.1 mM of DTT and EDTA.

Spinach leaf extract (*Spinacia oleracea*) was obtained from Dr. Elizabeth Weretilnyk and Dr. Peter Summers from the Department of Biology at McMaster University, Hamilton. The cell extracts from these leaves in the above buffer was made as described in Weretilnyk *et al.* 2001 [54]. *L. donovani* cells were kindly provided to us by Dr. Neil Reiner, Department of Medicine, University of British Columbia, Vancouver. The cells were sonicated in 50 mM Tris-maleate buffer pH 7.4 and 100 mM NaCl. The cell suspension was centrifuged and the extract was stored as described above.

5.6.2 Site-Directed Mutants of Adenosine Kinase

The cloning of full length CHO AK cDNA has been described in our earlier work [21]. The AK cDNA was subcloned into pET-15b (Novagen) expression vector with a (His)₆ tag at the N-terminal end as previously described [55]. Site directed mutagenesis at N239 and E242 were made by the use of 'Quikchange' site-directed mutagenesis kit (Stratagene) as described in earlier work [55]. The changes in the mutants N239Q, N239L, E242D and E242L were verified by DNA sequencing. After transforming BL-21(DE3) cells with the mutant plasmids, expression of the recombinant proteins was induced by the addition of 0.1 mM IPTG. The recombinant AK proteins from the WT and different mutant clones were purified by nickel affinity chromatography as previously described [49].

5.6.3 Adenosine Kinase Activity Assays

The AK activity was measured by means of a radioactive assay involving the conversion of ^3H -adenosine into ^3H -AMP as described earlier in our work [49;51;55]. The standard reaction mixture for AK activity determination of AK in cell extracts contained 50 mM Tris-maleate buffer pH 7.4, 10 μM adenosine (specific activity = 265mCi/mmol), 1 mM ATP and 1.94 mM MgCl_2 . The standard reaction mixture for the purified recombinant proteins contained 50 mM Tris-maleate buffer pH 6.2, 10 μM adenosine (specific activity = 265mCi/mmol), 1 mM ATP and 1.78 mM MgCl_2 . The lower pH was used to mimic the ischemic conditions. (2,8- ^3H)-adenosine (30.1 Ci/mmol) was obtained from DuPont Canada Inc. All other chemicals were of analytical reagent grade. Reactions were initiated by the addition of either 12 μg protein from cell extracts or 47 pmol purified AK to a final 300 μL volume of reaction mixture at 37°C. Initial velocities were determined by withdrawing 50 μL samples at various time intervals and adding these to 1mL of cold LaCl_2 in order to precipitate the radio-labeled AMP formed in the reaction. After keeping on ice for a minimum of 3 h, the precipitate was collected by filtration on a glass fiber filter and washed with 20 volumes of cold distilled water. The dried glass fiber filters were placed in 5 ml scintillation vials with aqueous scintillation fluid and the radioactivity was measured on a Beckman LS 7800 scintillator. Kinetic constants were determined by plotting the initial velocities against varying substrate concentrations using Graphpad Prism version 1.03.

Optimum activity for the *L. donovani* protein required a concentration of 2.88 mM MgCl_2 and fixed adenosine concentrations of 30 μM . For $K_m^{\text{Adenosine}}$ and K_m^{ATP}

determinations, the mutant enzyme N239Q required fixed concentrations of adenosine at 100 μ M and ATP at 100 mM. K_m^{ATP} for the mutant enzyme E242D required a fixed concentration of 20 μ M adenosine. In the absence of phosphate, the optimum concentration of ATP for E242D was 20 mM, in the presence of 20 mM PO_4 , the optimum concentration was 2 mM.

5.6.4 Molecular Modeling

Three dimensional coordinates for human AK (1BX4), ribokinase (1RKD) and *T. gondii* AK (1DGY) were obtained from The Protein Data Bank [56]. The coordinates were imported into Swiss-PDB viewer version 3.6b2 software, superimposed, and a structural alignment was performed. The RMS deviation of the backbone was then minimized. The 3D coordinates for M2 of the human AK structure and PO_4 of the ribokinase structure were then modeled into the active site of *T. gondii* AK (already containing coordinates for adenosine and AMP-PCP ligands), retaining H-bond distances and angles from residues in the original structures. No steric clashes between protein nor ligands were generated. Active-site diagrams were then reconstructed into two dimensions with CS ChemDraw Pro version 4.5.

5.7 Results

We have previously shown that PVI such as phosphate, arsenate and vanadate stimulated the activity and decreased the K_m of AK for adenosine from several mammalian sources (*vis.* Chinese hamster, Syrian hamster, beef liver and human) [49;51]

(and unpublished results). Recently bacterial RK, which is another member of the PfkB family, was also shown to be similarly affected by PVI [50]. In order to understand the role of PVI in the activity of AK, it was necessary at first to determine whether the requirement of PVI for activity was a common characteristic of AK from divergent sources, and then to identify the residues that may be responsible for this effect.

5.7.1 Effects of Pi on the Initial Velocity of AK from Diverse Sources

We have examined the effect of increasing PVI concentration on the activity of AK in cell extracts from plant, yeast, CHO and the parasitic organism *L. donovani*. Tables 5.1 and 5.2 show the effects of different concentrations of PVI on the kinetic parameters of AK from these sources. The PVI increased the maximum velocity in all cases, which was correlated with a substantial increase in K_i and decrease in K_m for adenosine. Since both adenosine and ATP are also metabolized to a certain extent by other enzymes present in cell extracts, the K_m values observed under such conditions should be regarded as apparent values. The results obtained in these studies are discussed below.

Similar to our earlier studies with purified AK from Syrian hamster and beef liver [49;51;52] and recombinant CHO [49], the activity of AK from CHO cell extracts was stimulated in the presence of PVI. The K_i for adenosine increases from 9.5 μM in the absence of PVI to 97.1 μM , 66.1 μM and 48.0 μM in the presence of 20 mM phosphate, arsenate and vanadate respectively. As shown for PO_4 , the decrease in K_i occurred in a concentration dependent manner. The maximum velocity increases and $K_m^{\text{Adenosine}}$

decreases upon the addition of increasing concentrations of PO_4 and in the presence of 20 mM arsenate and vanadate (Table 5.1). The apparent K_m for ATP was not affected in the presence of PVI and remained at approximately 0.15 mM under all conditions (Table 5.2). The effects of PVI on the spinach enzyme were very similar to the mammalian enzyme on the kinetic constants of $K_i^{\text{Adenosine}}$, maximum velocity and $K_m^{\text{Adenosine}}$ (Table 5.1). The K_m^{ATP} for this enzyme was also not altered in the presence and absence of PVI (Table 5.2).

The maximum velocity of the yeast (*S. cerevisiae*) AK was markedly increased upon the addition of PVI (Table 5.1). The $K_i^{\text{Adenosine}}$ for yeast AK is similar to the mammalian enzyme in the PVI-free form, but in the presence of 20 mM phosphate and arsenate, the concentration of adenosine necessary to invoke substrate inhibition was approximately 4-fold higher increase in comparison to the mammalian and plant AK. The apparent K_m of the enzyme for ATP remains constant under all conditions and approximately two fold lower than that of the CHO enzyme (Table 5.2).

The AK from *L. donovani* AK, as reported in the literature [41], is not inhibited by excess Mg^{2+} or high concentrations of adenosine. However, similar to AK from other sources, the addition of PO_4 as well as other PVI lead to a stimulation of its activity and a reduction in the K_m for adenosine (Table 5.1). In the presence of 20 mM arsenate and vanadate, some inhibition of enzyme activity was observed at high adenosine concentrations. The apparent K_m of the enzyme for ATP remains constant at approximately 0.050 mM under all conditions (Table 5.2).

Table 5.1 Steady-State Kinetic Parameters of Co-substrate Adenosine for AK in Cell Extracts

Enzyme	PVI (mM)	K_m^b (μM)	V_{max} ($\text{pmol s}^{-1}\mu\text{g}^{-1}$)	K_i (μM)	
CHO cells	0	32 ± 25	0.038 ± 0.025	9.5 ± 7.6	
	PO ₄	1	6.6 ± 2.2	0.077 ± 0.006	29 ± 4.2
		5	1.7 ± 0.20	0.102 ± 0.004	55 ± 7.4
		20	0.80 ± 0.10	0.108 ± 0.002	97 ± 9.2
	AsO ₄	20	4.9 ± 3.9	0.102 ± 0.005	66 ± 8.8
		VO ₄	20	7.8 ± 5.6	0.066 ± 0.005
Spinach	0	20 ± 7.4	0.026 ± 0.007	38 ± 17	
	PO ₄	1	4.6 ± 0.80	0.032 ± 0.003	77 ± 16
		5	1.3 ± 0.30	0.068 ± 0.005	82 ± 23
		20	0.90 ± 0.10	0.083 ± 0.003	120 ± 24
	AsO ₄	20	2.5 ± 0.60	0.080 ± 0.007	65 ± 16
		VO ₄	20	4.8 ± 1.1	0.045 ± 0.005
Yeast	0	26 ± 17	0.011 ± 0.006	18 ± 13	
	PO ₄	1	11 ± 2.0	0.057 ± 0.006	170 ± 53
		5	6.0 ± 0.70	0.114 ± 0.006	480 ± 190
		20	5.5 ± 0.80	0.123 ± 0.008	440 ± 200
	AsO ₄	20	11 ± 0.80	0.129 ± 0.005	240 ± 42
		VO ₄	20	12 ± 1.7	0.106 ± 0.009
<i>L. donovani</i>	0	13 ± 1.8	0.003 ± 0.0001	- ^a	
	PO ₄	0.1	3.0 ± 0.4	0.006 ± 0.0002	- ^a
		1	1.7 ± 0.1	0.023 ± 0.0003	- ^a
		5	0.8 ± 0.1	0.030 ± 0.0003	- ^a
		20	0.6 ± 0.1	0.034 ± 0.001	- ^a
	AsO ₄	20	4.3 ± 0.5	0.035 ± 0.002	220 ± 51
		VO ₄	20	6.7 ± 1.9	0.032 ± 0.001

^a Substrate inhibition not detected.

^b The K_m in cell extracts should be considered as apparent values.

Table 5.2 Steady-State Kinetic Parameters of Co-substrate ATP for AK in Cell Extracts

Enzyme	PVI (mM)	$Km^{apt\ b}$ (mM)	$Vmax$ ($\mu\text{mol s}^{-1}\mu\text{g}^{-1}$)
CHO cells	0	- ^a	- ^a
	PO ₄ 20	0.142 ± 0.010	0.114 ± 0.142
	AsO ₄ 20	0.151 ± 0.022	0.059 ± 0.151
	VO ₄ 20	0.149 ± 0.016	0.035 ± 0.149
Spinach	0	0.105 ± 0.014	0.012 ± 0.105
	PO ₄ 20	0.105 ± 0.004	0.072 ± 0.105
	AsO ₄ 20	0.109 ± 0.012	0.062 ± 0.109
	VO ₄ 20	0.101 ± 0.006	0.028 ± 0.101
Yeast	0	0.060 ± 0.005	0.004 ± 0.060
	PO ₄ 20	0.060 ± 0.008	0.092 ± 0.060
	AsO ₄ 20	0.060 ± 0.005	0.070 ± 0.060
	VO ₄ 20	0.057 ± 0.007	0.053 ± 0.057
<i>L. donovani</i>	0	- ^a	- ^a
	PO ₄ 20	0.049 ± 0.006	0.034 ± 0.049
	AsO ₄ 20	0.052 ± 0.004	0.032 ± 0.052
	VO ₄ 20	0.050 ± 0.003	0.026 ± 0.050

^a Activity too low to calculate constants

^b The Km of ATP in cellular extracts should be considered as apparent values

5.7.2 Effects of Pi on Site-directed Mutants Altered in the NXXE Motif.

Our studies of the AK and RK structures indicate that the N239 and E242 residues in CHO AK, which are part of the conserved NXXE motif, interacts with PO_4 and Mg^{2+} ions. To determine the importance of these interactions for the activity of AK, we have made specific alterations in these residues of CHO cDNA by site-directed mutagenesis. The mutants that we have generated consisted of replacing N239 with either glutamine (N239Q) or leucine (N239L) and replacing Glu242 with either aspartic acid (E242D) or leucine (E242L). The recombinant WT and the mutant AK proteins were purified and their response to PO_4 and Mg^{2+} were studied. Preliminary studies performed at both pH 7.4 and 6.2 showed similar effects. However, the magnitude of these effects was higher at pH 6.2 than at pH 7.4 (data not shown). Therefore, further studies with these proteins were carried out at pH 6.2.

Figures 5.3 and 5.4 show the secondary plots of initial velocity versus substrate concentration for WT AK and two of the mutants, N239Q and E242D. The other two mutants N239L and E242L showed negligible AK activity under the standard assay conditions and their results will be discussed later. As seen from Figures 5.3 and 5.4, WT AK shows negligible activity in the absence of PO_4 and activity is greatly stimulated upon the addition of PO_4 . In contrast, the N239Q mutation results in a protein which shows substantial activity in the absence of PO_4 and there is no appreciable change in activity upon the addition of PO_4 (Figures 5.3 B and 5.4 B). Further, in contrast to the WT enzyme which showed inhibition at higher concentrations of substrates, the activity

from N239Q mutant was not inhibited at high concentrations of either adenosine or ATP. The mutant protein E242D also showed interesting changes in its response to PO_4 . This protein also exhibited substantial AK activity in the absence of PO_4 , and was stimulated only about 3 fold in the presence of 20 mM PO_4 . The effects of these mutations on the properties of AK can be best understood by the values of various kinetic constants for the WT and the mutant enzymes (Tables 5.3 and 5.4).

As seen from Table 5.3, the $K_m^{\text{Adenosine}}$ for the N239Q enzyme in the absence of phosphate is approximately 3.5 fold higher than that of the wild-type enzyme. However, in contrast to the WT, where $K_m^{\text{Adenosine}}$ is greatly reduced in the presence of PO_4 (more than 20 fold), the K_m for the mutant enzyme was not significantly affected by phosphate. As a result, the k_{cat}/K_m ratio for the N239Q protein is about 14 fold higher than WT in the absence of PO_4 . However, in the presence of PO_4 , the k_{cat}/K_m ratio of the mutant protein is about 20 fold lower than that of WT. Another significant change seen for the N239Q protein was that the K_m for ATP is about 200 fold higher than seen for WT AK. The K_m^{ATP} for the mutant protein was slightly reduced in the presence of PO_4 (Table 5.4).

The E242D protein was more active than the WT protein in the absence of phosphate. The k_{cat} for this protein is increased about 2-3 fold in the presence of

Table 5.3 Steady-State Kinetic Parameters of Co-substrate Adenosine for Wild-type and Site-directed Mutants.

Enzyme	PVI (mM)		K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\text{M}^{-1}\text{s}^{-1}$)	K_i (μM)
Wild-type	PO ₄	0	19 ± 7.7	0.08 ± 0.02	4.16 × 10 ³	22 ± 9.9
		20	0.80 ± 0.10	1.18 ± 0.04	1.47 × 10 ⁶	76 ± 9.4
N239Q	PO ₄	0	65 ± 6.4	3.73 ± 0.26	5.78 × 10 ⁴	250 ± 42
		20	69 ± 12.7	3.85 ± 0.51	5.57 × 10 ⁴	220 ± 64
E242D	PO ₄	0	6.0 ± 2.1	0.23 ± 0.04	3.79 × 10 ⁴	66 ± 26
		20	11 ± 3.5	0.80 ± 0.20	7.42 × 10 ⁴	18 ± 5.7

Table 5.4 Steady-State Kinetic Parameters of Co-substrate ATP for Wild-type and Site-directed Mutants.

Enzyme	PVI (mM)		K_m (mM)	k_{cat} (s^{-1})	K_i (mM)
Wild-type	PO ₄	0	0.17 ± 0.03	0.03 ± 0.01	9.6 ± 2.2
		20	0.16 ± 0.01	1.14 ± 0.03	20 ± 2.1
N239Q	PO ₄	0	31 ± 7.6	2.57 ± 0.41	280 ± 170
		20	22 ± 4.3	2.50 ± 0.30	420 ± 270
E242D	PO ₄	0	2.7 ± 0.47	0.15 ± 0.01	110 ± 26
		20	0.25 ± 0.07	0.26 ± 0.02	26 ± 8.0

Figure 4.1. The Effect of Pentavalent Ions on K_m D-ribose.

The initial velocity is plotted against increasing concentrations of D-ribose. (A)

Phosphate: 0 (■); 0.5 mM (◆); 1 mM (▼); 5 mM (●) and 20 mM (▲). (B) Arsenate: 0

(■); 0.5 mM (◆); 1 mM (▼); 5 mM (●) and 20 mM (▲). (C) Vanadate: 0 (■); 1 mM (▼);

5 mM (●) and 20 mM (▲). (D) Activity in the absence (■) and presence (▲) of 20 mM

phosphoenol pyruvate. The error bars shown in all figures are derived from the standard error of the initial velocity, and they do not represent results from replicate experiments.

Figure 5.3.

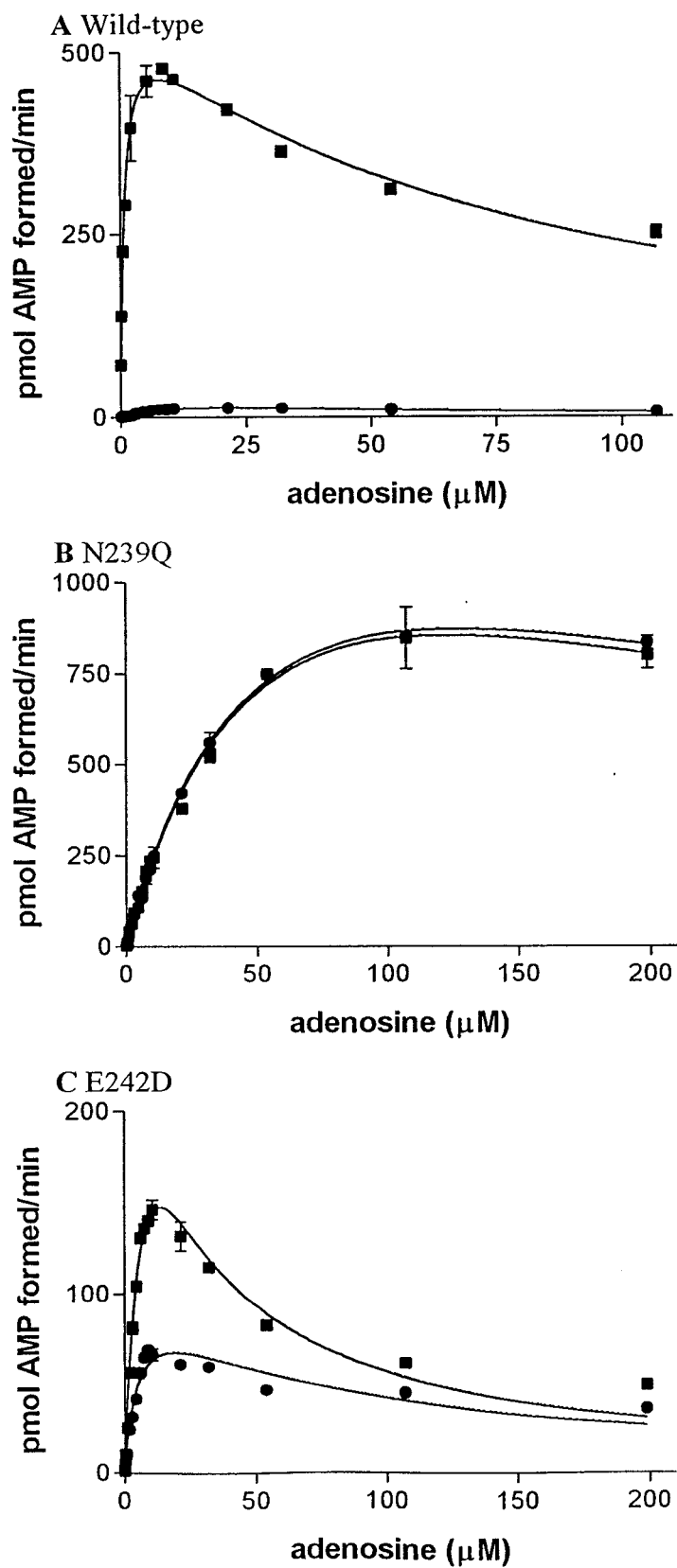
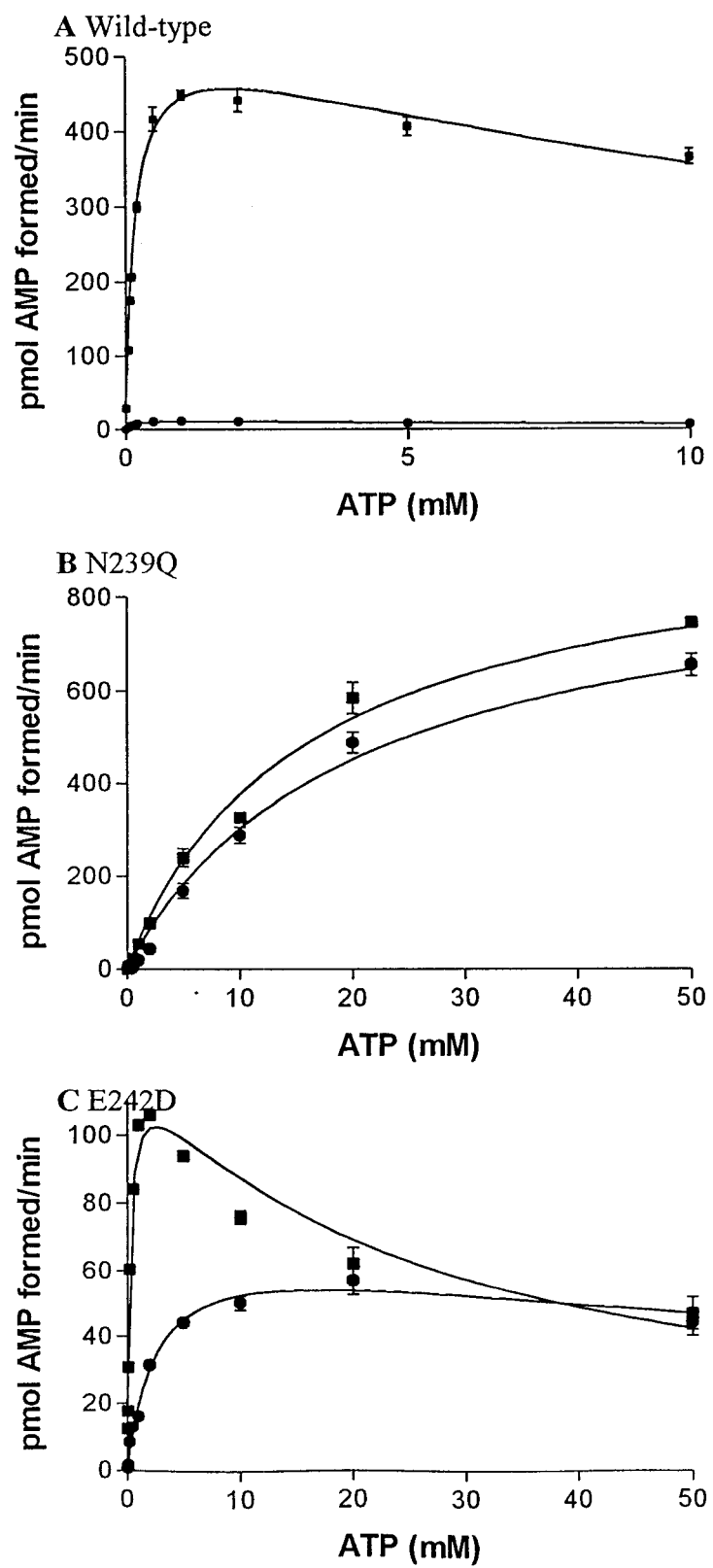


Figure 5.4 Effects of Pentavalent Ions on the Initial Velocity and K_m for ATP.

Initial velocity is plotted against ATP concentrations for wild-type and mutant AK. (A) wild-type in the absence of pentavalent ions (●) and in the presence of 20 mM phosphate (■); (B) N239Q in the absence (●) and presence of 20 mM phosphate (■); (C) E242D in the absence (●) and presence of 20 mM phosphate (■). Concentrations of reagents are as described in Experimental Procedures.

Figure 5.4.



phosphate (Table 5.3). The K_m for adenosine is slightly higher and $K_i^{\text{Adenosine}}$ is also somewhat lower in the phosphate bound form (Table 5.3). The most interesting effect of this mutation is seen in the K_m for ATP (Table 5.4), which is found to be about 15 fold higher in the absence of phosphate and then reduced by a factor of 10 in the presence of phosphate compared to WT. This is the only enzyme for which the K_m for ATP is significantly altered in the presence of PO_4 (Table 5.4). Another interesting feature of this mutant is that K_i^{ATP} is decreased approximately 3 fold in the presence of phosphate, to a value similar to that seen for WT AK under similar conditions (Table 5.4).

5.7.3 Mg^{2+} Ion Dependency of the WT AK and Various Mutants Affected in the NXXE Motif.

We have examined the activity of wild-type CHO AK as a function of free Mg^{2+} in the presence and absence of phosphate so that any changes in the mutants could be determined. The calculation of free Mg^{2+} was performed as previously described by Fabiato and Fabiato [57]. In solution at pH 6.2, at 1 mM ATP and 0.5 mM MgCl_2 (0.15 mM free Mg^{2+}), ATP is complexed to nearly 35% by magnesium. Under these conditions, there was no detectable wild-type activity. Above 0.75 mM MgCl_2 (0.26 mM free Mg^{2+}) the initial velocity of wild-type increases hyperbolically to maximal at 1 mM free Mg^{2+} (Figure 5.5). Above concentrations of 1mM, free Mg^{2+} increasingly inhibits activity. In the presence of 20 mM phosphate the apparent $K_{\text{act}}^{\text{Mg}^{2+}}$ for WT AK is not altered (0.4 mM), however the apparent $K_i^{\text{Mg}^{2+}}$ is increased by about 15 fold (Table 5.5).

The initial velocity of various mutants was examined as a function of free Mg^{2+} . The magnitude of substrate inhibition by adenosine, and to a lesser extent by

Mg^{2+}/ATP^{4-} , are influenced by the concentration of free Mg^{2+} [10;58;58] all of which are inter-related with the pH of the assay [8]. It should be noted that these experiments were carried out at fixed substrate concentrations (10 μ M adenosine and 1 mM ATP) which were optimal for the wild-type enzyme at 1 mM free Mg^{2+} . As a result, the derived kinetic constants for the wild-type and mutant enzymes are likely sub-optimal and should be considered as apparent values, included for comparative purposes only.

N239 makes one close contact with M2 (3.5Å) in the structure of the human enzyme and one close contact with O3 of phosphate (4.0Å) in the RK structure (Figures 5.1 A and B). This residue also makes close contacts with a non-bridging oxygen of the β -phosphate of ADP (4.3 Å) and AMP-PCP (3.5 Å) in the structure of RK and *T. gondii* AK respectively (Figures 5.1 B and C). For the N239Q mutant protein, the $kcat^{Mg^{2+}}$ and $Kact^{Mg^{2+}}$ values in the presence of PO_4 were very similar to the WT AK. However, in the absence of PO_4 , N239Q has a $kcat^{Mg^{2+}}$ value 4 times lower and $Kact^{Mg^{2+}}$ slightly higher than wild-type. Under these conditions, this enzyme is not inhibited by high concentrations of Mg^{2+} (Table 5.5). For the E242D mutant, the $Kact^{Mg^{2+}}$ is higher in the absence of phosphate but is decreased to a value comparable to the WT upon the addition of PO_4 . Both the N239L and E242L mutants displayed only weak activity even at very high concentrations of Mg^{2+} . Hence, for clarity sake, the activity data for these mutants are presented by percent maximal activity for each protein studied. Due to the low activity seen with these mutants, the errors in various constants are high for these proteins (Table 5.5). However, for both these mutants, the $Kact^{Mg^{2+}}$ was found to be between 10-25 fold higher than the WT enzyme both in the presence and absence of PO_4 and the activity was significantly enhanced by phosphate.

Figure 5.5 Magnesium ion dependencies of WT and various mutant AK

Percent maximal velocities are plotted against free magnesium concentrations in the absence (A) and presence (B) of 20 mM phosphate for WT (■), N239L (▼), N239Q (▲), E242L (●), and E242D (◆). Kinetic parameters determined from curve fitting are reported in Table 3.

Figure 5.5.

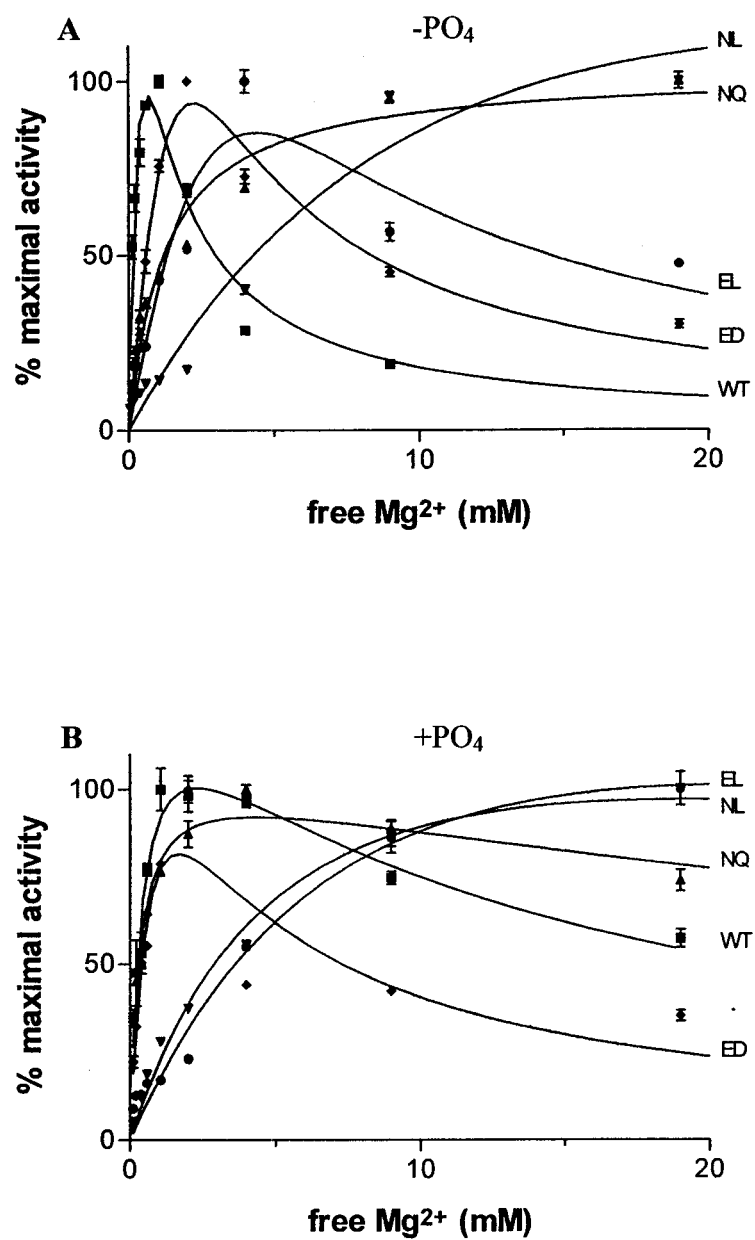


Table 5.5 Steady-State Kinetic Parameters of Mg²⁺ for Wild-type and Mutant AK.

Enzyme	PO ₄ (mM)	<i>K_{act}</i> (mM)	<i>k_{cat}</i> (s ⁻¹)	<i>K_i</i> (mM)
Wild-type	0	0.60 ± 0.50	0.068 ± 0.036	0.70 ± 0.50
	20	0.40 ± 0.10	0.159 ± 0.016	12 ± 3.5
N239Q	0	1.3 ± 0.20	0.016 ± 0.001	- ^a
	20	0.30 ± 0.10	0.146 ± 0.008	55 ± 14
N239L	0	16 ± 10	0.001 ± 0.0004	53 ± 50
	20	4.4 ± 0.6	0.008 ± 0.001	490 ± 420
E242D	0	8.3 ± 16	0.082 ± 0.148	0.60 ± 1.2
	20	0.80 ± 0.70	0.171 ± 0.089	3.5 ± 3.1
E242L	0	14 ± 40	0.0002 ± 0.001	1.4 ± 4.0
	20	11 ± 4.7	0.003 ± 0.001	41 ± 23

^a Substrate inhibition not detected

5.8 Discussion

Adenosine kinase is a key enzyme in the metabolism of adenosine which shows potent pharmacological activity [33;59-61]. Although AK has been studied for more than 30 years, its mechanism of action is still not completely understood and novel and important regulatory features of this enzyme continue to be uncovered. One unexpected feature of AK function discovered in our work a few years ago was that its catalytic activity required the presence of PVI such as phosphate, arsenate or vanadate. These studies also provided evidence that the PVI were not consumed nor directly participated in the reaction catalyzed by AK. All previous studies on the effect of PVI on AK have been carried out with the enzyme derived from mammalian sources (49;51). Thus, an important question was whether the PVI dependency of AK was restricted to the mammalian enzyme or whether it constituted a conserved intrinsic property of the enzyme. Results presented here now show that the pentavalent ions caused a marked enhancement in the activity of AK from various sources including mammals, plants, yeast and the protist species *Leishmania donovani*. In all cases, phosphate and other PVI have been found to exert their effects primarily by decreasing the K_m for adenosine, and by alleviating the inhibition caused by high concentrations of the co-substrates. The AK from *L. donovani*, which is not inhibited at high adenosine and magnesium concentrations, is also similarly stimulated by PVI, providing evidence that the pentavalent ion dependency is an intrinsic characteristic of the enzyme from various sources. A number of cations and anions such as K^+ , Na^+ , Fe^{2+} , Cu^{2+} , Ca^{2+} , Zn^{2+} , acetate,

sulfate, sulfite, nitrate and carbonate have previously been investigated, however only pentavalent ions have been found to stimulate AK activity [51;52].

AK is a member of the PfkB family of carbohydrate kinases. We have recently shown that similar to AK the catalytic activity of RK, another member of the PfkB family, was also strongly stimulated by PVI, which acted by lowering the K_m of the enzyme for D-ribose. In addition to AK and RK, three other enzymes involved in the transfer of phosphate from ATP to a sugar derivative (*viz.* 6-phosphofructo-2-kinase (EC 2.7.1.105) [62], phosphoribosylpyrophosphate synthetase (EC 2.7.6.1) [63] and 6-phosphofructokinase (EC 2.7.1.11) [64] have been reported to show a similar dependence on PO_4 for activity. It is thus likely that PVI dependency is a common characteristic of the PfkB family of proteins as well as some other carbohydrate kinases.

The mechanism by which PVI stimulates, or are required for the activity of AK and RK (or the other above mentioned enzymes), is presently not understood. However, potentially useful information in this regard was provided by the crystallographic data of AK and RK. These data indicated that asparagine 239 and glutamic acid 242 in the CHO AK sequence, which are part of the conserved NXXE motif found in both these enzymes as well as various other PfkB family members, make close contacts with Mg^{2+} (M2) and PO_4 ions in their respective structures. To determine whether these residues indeed play an important role in the interaction of PO_4 and Mg^{2+} ions, these residues were replaced with other amino acids and the effect of these alterations on the activities of these enzymes and their response to PO_4 and Mg^{2+} ions was determined. Results of these

studies strongly support the contention that the NXXE motif identified by us plays an important role in the effects of these ions on the catalytic activity of AK.

Of the two conserved amino acid residues that are part of the NXXE motif, the asparagine at position 239 has an uncharged polar side-chain. It was mutated to either similarly charged glutamine, which is one carbon unit longer, or to the non-polar amino acid leucine, which is similar in size to asparagine. N239 makes close contacts with a non-bridging oxygen of the β -phosphate of ADP (4.3 Å) and AMP-PCP (3.5 Å) in the structure of RK and *T. gondii* AK respectively (Figures 1 B and C). If N239 contributed to catalysis solely by assisting in the binding of ATP, then it is reasonable to assume that the mutation of it would mainly affect the K_m for ATP. However, the N239Q mutant showed dramatically higher K_m values for both adenosine and ATP (particularly in the presence of PO_4) and the stimulatory effect of phosphate on the catalytic activity of the enzyme was entirely eliminated. The N239L mutant showed very weak and inefficient AK activity, however, it required much higher concentrations of Mg^{2+} for optimal activity. The side chain of N239 makes one close contact with M2 (3.5 Å) in the human AK structure and a close contact with O3 of PO_4 (4.0 Å) in the RK structure (Figures 1 A and B). The replacement of this residue with the nonpolar leucine residue, which is unable to form these contacts, was expected to result in a large decline in AK activity and altered Mg^{2+} requirement. Although the exact mechanistic effect of the N239 residue in AK activity remains uncertain, it is quite clear that changes in this residue have a major impact on both phosphate stimulation and Mg^{2+} requirement.

The glutamic acid residue at position 242 interacts with two coordinated waters of M2 ($<2.8\text{\AA}$) in the human AK structure and also forms two contacts with the oxygens of PO_4 (2.8\AA and 4.2\AA) in the structure of RK (Figures 1A and B). The side-chain of E242 also forms a hydrogen bond with the main-chain nitrogen of N239. The negatively charged residue E242 was replaced with either aspartic acid, which is one carbon unit shorter but retains the negative charge, or with leucine which is nonpolar and similar in size to aspartic acid. E242 does not directly interact with ATP or adenosine yet in the absence of phosphate, the E242D mutant showed an increase in K_m for both ATP and Mg^{2+} as well as higher K_i for ATP compared to wild-type. However the addition of phosphate evokes an increase in K_m and a decrease in K_i for adenosine. The presence of phosphate has the opposing effect of decreasing the K_m^{ATP} and apparent $K_m^{\text{Mg}^{2+}}$. These opposing effects of the E242D mutation suggests that the role of residue E242 is complex and not limited to H-bonding with N239 for the optimal binding of ATP. The E242L mutation leads to an enzyme with greatly reduced activity and much higher apparent K_m for Mg^{2+} .

The observed changes in the kinetic properties of AK upon mutation of N239 and E242 strongly suggest that these residues are involved in controlling AK activity through the binding of phosphate and magnesium ions. To account for these observations and the various ligands found at the active sites of RK and human and *T. gondii* AK, a model as to how these residues are linked and interact is suggested (Figure 5.6). The construction of the model is described in the Experimental Procedures section. The residue numbering of this model is based on the amino acid sequence of CHO AK. M2 from the human AK

structure and PO₄ from the RK structure were modeled into the active site of *T. gondii* AK which contains coordinates for adenosine, AMP-PCP and M1. The distances of M2 and PO₄ to the protein and bond angles seen in the original structures were retained, and the modeling of these ligands does not interrupt any existing H-bond interactions.

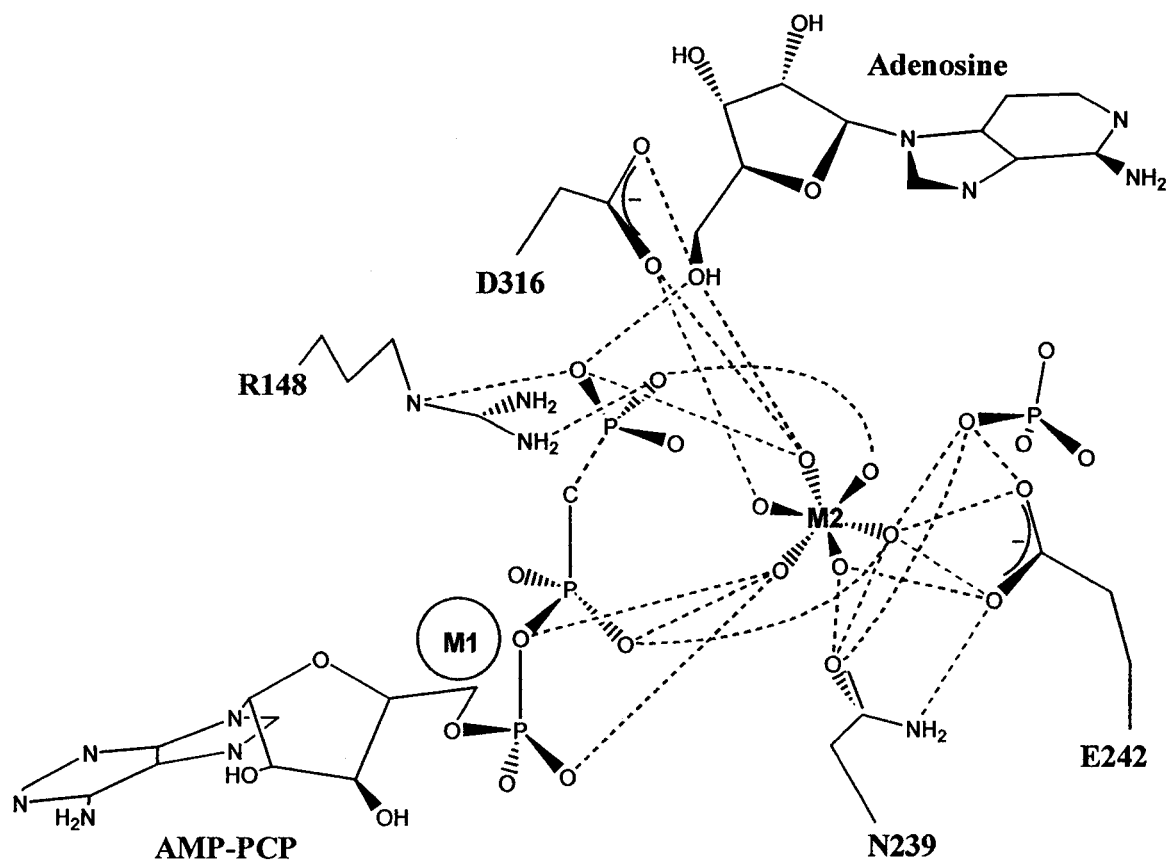
Crystallographic water molecules at the active site of *T. gondii* were also retained. Four of these water molecules superimpose with the coordinated waters of M2, and three of these waters are found in similar 3D space at the active site of RK.

As seen in the 3D structure of human AK, the coordinated waters of M2 forms one H-bond contact with the 5'-hydroxyl of adenosine, and three to the carboxylate of the putative catalytic residue Asp316. The close contacts of M2 to N239 and E242 are also retained. In this model, M2 forms a close contact with the bridging oxygen between α and β phosphates, as well as a number of H-bond contacts with non-bridging oxygens of α , β and γ -phosphates of AMP-PCP. As seen in the original RK structure, the modeled phosphate retains similar distances to residues N239 and E242. In this model, PO₄ forms a hydrogen bond with a coordinated water of M2 as well as a close contact with a non-bridging oxygen of the β -phosphate of AMP-PCP (not shown).

Figure 5.6 A Model Showing the Interactions of Substrates and Ligands at the Active Site of Adenosine Kinase.

The ligands adenosine, AMP-PCP and M1 are found in the original *T. gondii* structure. M2 of the human AK and PO₄ of ribokinase were easily modeled into the active site of *T. gondii* with Swiss PDB Viewer version 3.6B2. No steric clashes between existing residues or ligands were generated. The 3D image was then translated into 2D with CS ChemDraw Pro version 4.5. The side-chains of CHO AK Arg148, Asn239, Glu242 and Asp316, which form an intricate H-bond network with ligands, are absolutely conserved among AK. Two main-chain contacts from PO₄ to conserved large domain residues Ser214 and Pro216 are not shown in this substrate-bound, closed conformation of AK.

Figure 5.6.



The small domain residue arginine 148 is shown. In the original structures of *T. gondii* AK, this residue is translocated 13.7Å towards the active site upon the binding of adenosine, then rotates into the active site upon the binding of AMP-PCP, forming two hydrogen bonds with non-bridging oxygens of the γ -phosphate (not shown). Therefore, it is thought that this residue is responsible for compensating negative charges which develop during the transfer of the phosphoryl group from ATP to adenosine [46]. We have included this residue in our model as it forms two contacts less than 3.5Å with the coordinated waters of M2 (not shown). PO₄ also makes three contacts with Arg148 through water molecules (not shown).

Biochemical data for AK from a variety of sources presented here, and for ribokinase [50] suggest that the effects of pentavalent ions is two-fold. Firstly, the addition of pentavalent ions evokes a reduction in *K_m* for the first co-substrate to bind. A number of sequential conformational changes are required for enzyme turnover of PfkB proteins [46;48]. As both substrates and products contain identical features, PVI must facilitate the both the formation of the active site pocket as well as reduce the non-specific binding of ligands. Secondly, large increases in *K_i* for adenosine, ATP and Mg²⁺ for AK are seen in the presence of PVI. The present model shows that the electrophilic properties of pentavalent ions may assist in the rearrangement of charges at the active site to facilitate product release and/or restoring the resting state of the enzyme. M2 may be involved in the correct positioning of the arginine residue for the rearrangement of electrical charges which occur upon enzyme turnover.

The model suggests that PO_4 and M2 bound to the NXXE site are within correct distances to the active site to facilitate the productive binding of substrate and product release. Site directed mutagenesis of the NXXE motif clearly results in enzymes with greatly altered phosphate and magnesium requirements. The model also indicates that M2, located at the NXXE motif, is within H-bonding distances to facilitate the binding of ATP, as proposed for other phosphotransferases which require a second magnesium for optimal activity [65-68].

While we propose that the results from the mutagenesis studies arise from altered K_m and K_i for co-substrates, it is possible that M2 and PO_4 are involved in altering the rate-limiting step for enzyme turnover. High concentrations of Mg^{2+} have been shown to decrease the rate-limiting step of ADP release for other phosphotransferases [66]. Our results provide evidence that phosphate binds near the active site affecting the binding of substrate. This has set the stage for further studies to determine the rate-limiting step of AK and if this is affected in the presence of phosphate.

5.9 Acknowledgments

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5.10 References

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5.11 Appendix

Figure 5.7 Effect of Pentavalent Ions on the Activity and K_m for Adenosine for Various AK from Cell Extracts

Initial velocity of AK from cellular extracts is plotted against adenosine concentrations for A) Chinese hamster ovary; B) yeast, C) spinach; and D) *L. donovani*, in the absence (●)¹ and presence of 20 mM phosphate (■), arsenate (▲) and vanadate (◆).

¹ In some cases, activity in the absence of phosphate was too low to accurately determine initial velocity.

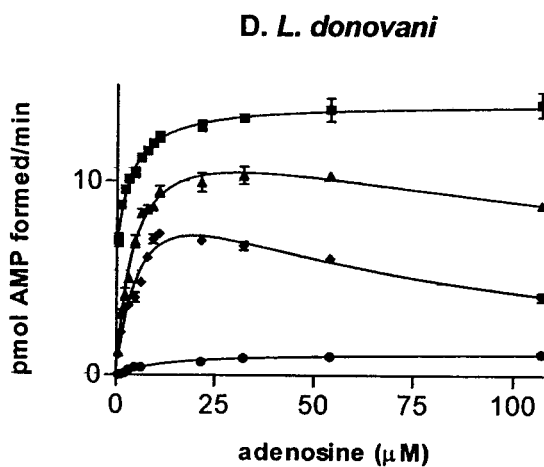
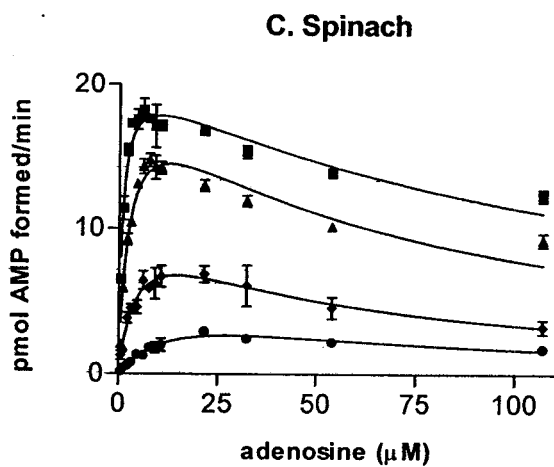
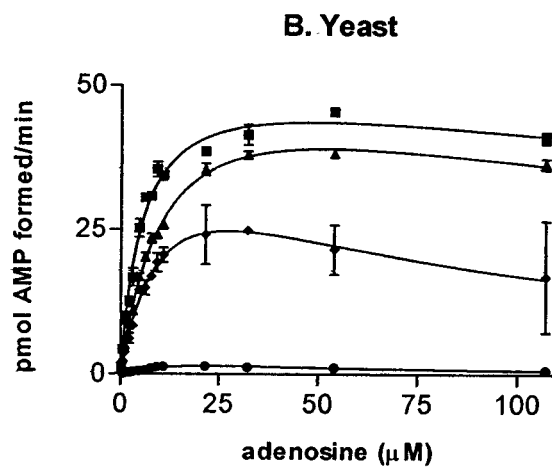
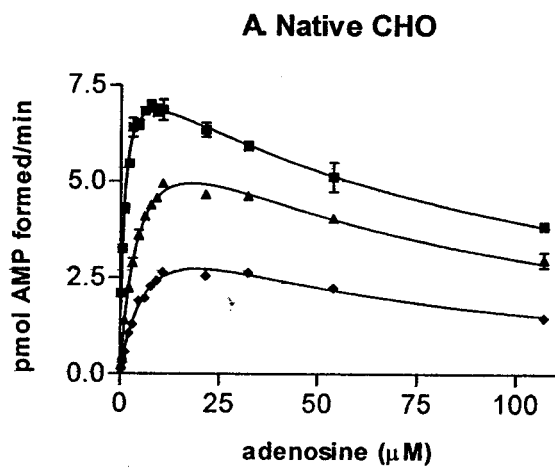
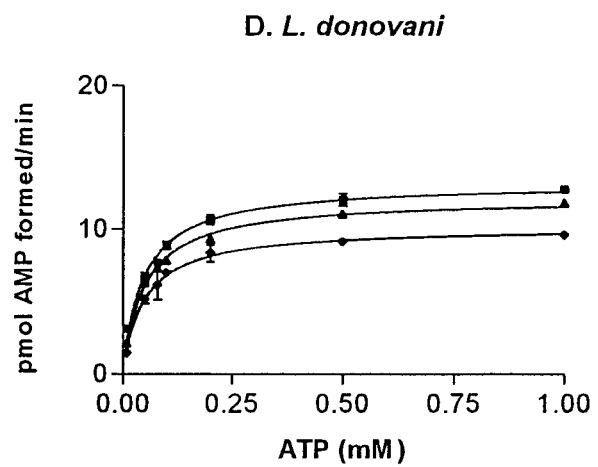
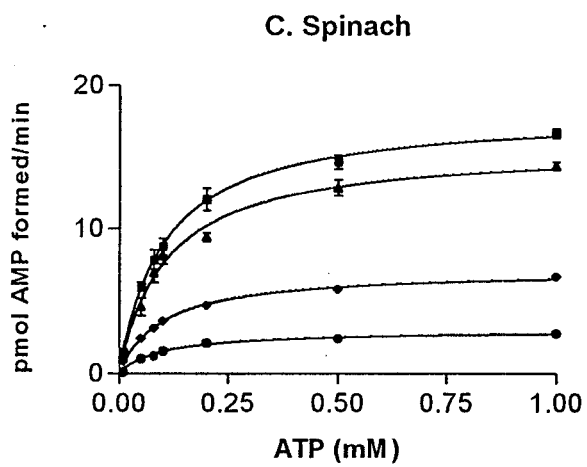
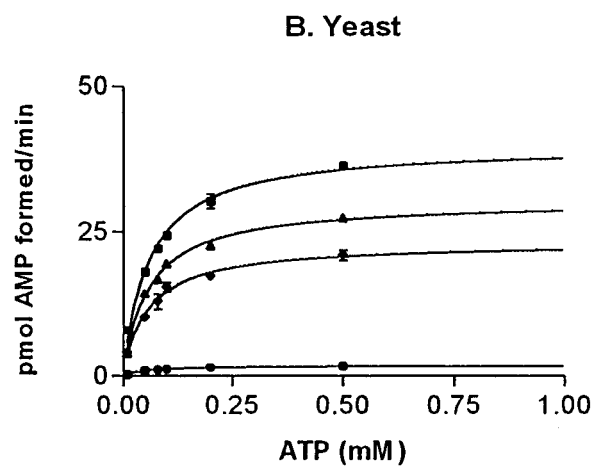
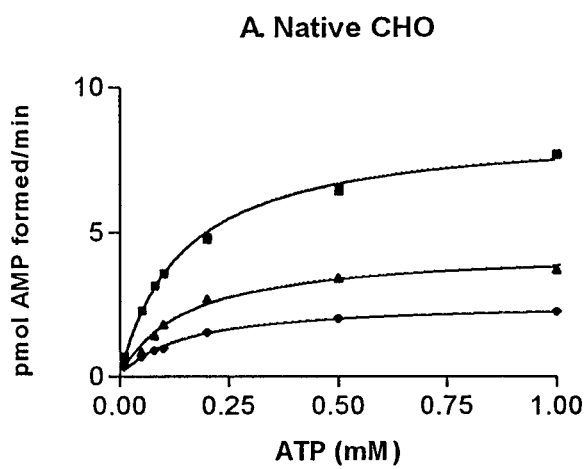


Figure 5.8 Effect of Pentavalent Ions on the Activity and K_m for ATP for Various AK from Cell Extracts

Initial velocity of AK from cellular extracts is plotted against ATP concentrations for A) Chinese hamster ovary; B) yeast, C) spinach; and D) *L. donovani*, in the absence (●)¹ and presence of 20 mM phosphate (■), arsenate (▲) and vanadate (◆).

¹ In some cases, activity in the absence of phosphate was too low to accurately determine initial velocity.



Chapter 6. Discussion and Conclusions

6.1 Discussion and Conclusions

Due to the accumulating evidence that adenosine behaves as an endogenous inhibitor of many disease processes through interaction with cell surface receptors, adenosine based pharmaceuticals continue to be explored. However, receptor agonists have been found to show side effect liabilities. Recently, the inhibition of AK has moved to the forefront of research as a method of increasing the endogenous concentration of adenosine with decreased side effects. A large number of AK inhibitors have shown to exert clinical utility in animal models of inflammation, nociception and seizures. Though the activity of AK has been studied for years, the exact mechanism of activity remains controversial. Thus, continued exploration of the structure and function of AK will further assist in the design of compounds to be used as pharmaceutical agents.

The focus of this thesis was to answer questions as to some novel properties of AK. The first focus being to examine the effect of inorganic phosphate on the kinetic parameters of mammalian AK, determine if this effect is conserved for AK from a variety of species, and to establish if phosphate also influences the activity of bacterial homologue ribokinase. Secondly, the importance of residues at the N-terminus was investigated. Adjunct issues regarding the variety of 'optimal' assay conditions for studying the enzyme activity from a variety of sources are also addressed.

The work presented in Chapter 2 was based on the observation that pentavalent ions could stimulate activity and reduce the K_m for adenosine [1]. Detailed analysis of

this phenomena revealed that not only is the V_{max} increased and K_m decreased, but the K_i for adenosine was increased as well. These studies also showed that phosphate exerted a larger effect at more acidic pH than at physiological pH values. Exploration of scientific literature revealed that the activity of AK is an important modulator of adenosine concentrations during ischemia and reperfusion, in an environment where local acidosis occurs.

During the development of a system to overexpress and purify AK from CHO cDNA in *E. coli*, it was realized that due to a sequence compression, the coding sequence of AK was indicated to be 27 residues shorter than actuality. The truncated version of the protein showed no activity in bacterial cell extracts, and could not be purified under native conditions. Sequence analysis of this truncated protein revealed no similarity to any other protein sequences published nor deposited in data banks. Upon sequencing the second strand of CHO cDNA, the compression was identified and the complete cDNA was ligated into an expression vector. Active CHO AK could be easily purified under native conditions with and without a 6-histidine tag at both the N- and C-terminus. This suggested that both termini were found on the surface of the protein and additional residues would not affect activity. In addition, survey of the literature revealed that the greatest variability in the amino acid sequence of AK from a variety of species could be found at the N-terminus. This prompted the investigation of the function of residues at the N-terminus.

Thus, the second focus of the work in this thesis was to determine the importance of residues at the N-terminus are presented in Chapter 3. The systematic deletion of

residues at the N-terminus was undertaken, and the activity and thermal stability of the resulting proteins was performed. As we had an efficient system for expressing AK in *E. coli* with either a C- or N-terminal histidine tag, the residues at the C-terminus were similarly studied. During the course of this work, the three-dimensional structure of human AK bound with two adenosine molecules was published, one adenosine site near the residues at the N-terminus and the second near residues at the C-terminus. Rather than obviate the deletion studies, the structural data assisted in the assignment of structural importance of the residues in question. It became quite clear that the first 16 residues of the N-terminus could sustain large variability as their deletion did little to affect activity, thermal stability or K_m for co-substrates. Deletion of residues downstream up to residue 27 resulted in proteins with increasing thermal instability, decreasing activity and slightly higher K_m for adenosine. The K_m for ATP remained relatively unchanged. Residues 17-27 are important for the stability of the first β -sheet structure of the large domain. Immediately following this region is the first β -structure of the small domain. The residues which compose this second β -strand are involved in adenosine binding. At the time of these studies, it was unclear which of the two adenosine molecules found in the structure of human AK represented the true adenosine binding site. The N-terminal deletion studies suggested that the K_m for adenosine was only slightly increased.

Systematic deletion of residues at the C-terminus were begun in hopes that these mutants were greatly altered in K_m for ATP. None of the residues at the C-terminus could be deleted without greatly decreasing activity and thermal stability. The K_m for

adenosine increased about two-fold, and the K_m for ATP increased approximately 5-fold for the largest active deletion compared to the wild-type enzyme. Inspection of the 3D structure showed that the residues at the C-terminus were involved in a hydrogen bonding network which stabilized an unconventional P-loop structure, suggesting that substrate binding site near the C-terminus represented the ATP site. Recent crystallographic data of *Toxoplasma gondii* AK bound with a variety of ligands has confirmed this hypothesis [2].

In 1996, the cloning of human AK was reported, and similarity to microbial ribokinases and fructokinases within two small motifs was identified [3]. These microbial sugar kinases belonged to the PfkB family of carbohydrate kinases [4;5]. Accordingly, AK was included into this family. Another member of the PfkB family was bacterial ribokinase. The 3D structure of RK was reported in 1997 [6], and the first insights into the structure of this family was revealed. The enzyme was co-crystallized with a phosphate group bound near the active site, though far enough away as not to represent the γ -phosphate position of ATP. Later that year, the structure of AK was published [7], revealing a strong similarity to RK. Though the amino acid sequence similarity was less than 30%, the residues at the active site were conserved in 3D space. An aspartic acid residue was found positioned near the 5'-hydroxyl group of the sugar moiety such that it was concluded that this residue may act as the catalytic base for both RK and AK. A magnesium ion with 6-coordinated water molecules was also shown near the active site of AK, similar to the phosphate found in the RK structure.

The structural similarities prompted first the investigation as to study the effects of mutating this putative catalytic base in AK, and second to determine if the activity of

RK is affected by pentavalent ions. As shown in Chapter 3, it was found that mutation of the putative catalytic base to either glutamic acid, asparagine and glycine, results in nearly complete loss of activity. Therefore, it was determined that this residue is instrumental for the activity of AK.

Biochemical investigation to determine if RK activity is affected by pentavalent ions ensued. The work presented in Chapter 4 reveals that RK, similar to AK, experiences stimulation of activity and a decrease in K_m for the phosphate accepting substrate in the presence of increasing concentrations of pentavalent ions. Though RK is not inhibited by high concentrations of ribose, similar to AK, it is inhibited by high concentrations of free magnesium and ATP. A survey of literature regarding the activity of other PfkB family members also reveals a number of the proteins are inhibited at high free magnesium and ATP concentrations. The similar effect of pentavalent ions on two distantly related proteins may suggest a common mechanism of activity for enzymes of this class.

In the 3D structures, phosphate was bound near the active site of RK, and magnesium was similarly bound to AK. Both molecules interacted with 2 residues which are conserved in 3D space. Sequence alignment of a number of PfkB proteins revealed that this NXXE motif is highly conserved. Consequently, these residues were mutated, and the activity of the resulting proteins were studied and presented in Chapter 5. N239 was altered to glutamine or lysine. Mutation to glutamine resulted in a protein which is not inhibited by high concentrations of adenosine, ATP nor magnesium. However, the activity of N239Q was not stimulated in the presence of phosphate, nor is the K_m for co-

substrates altered. N239L mutant showed a significant decrease in activity and altered Mg^{2+} requirement. E242 was mutated to aspartic acid and lysine. The presence of phosphate did stimulate the activity of the E242D protein, though the K_m for adenosine was not significantly altered. However, the concentration of co-substrates and magnesium necessary to evoke inhibition and the K_m for ATP was greatly decreased. Mutation of E242 to lysine required a much higher K_m for Mg^{2+} than the wild-type enzyme. The results of NXXE mutagenesis studies suggest that both N239 and E242 play an important role for magnesium binding and requirement. So to are these residues implicated in the phosphate effect seen for the wild-type enzyme. The results of replacement of either N239 and E242 with lysine further suggests that a second magnesium ion, different from that which is required for ATP binding, may be important for regulating AK activity.

Lastly, the study of AK activity in the past has been greatly different among enzyme sources in the choice of buffering systems, the pH of study, and the determination of optimal magnesium to ATP concentrations. As a result, it is difficult to compare the activity of enzymes from different species. In order to compare the activities of divergent AK, maximum velocity and K_m determinations for co-substrates of AK from cell extracts of CHO, spinach, yeast and *L. donovani* were performed under similar conditions. The activities of all enzymes studied were found to be stimulated in the presence of pentavalent ions. The K_m for adenosine was decreased for all enzymes studied, suggesting that the effect of phosphate is an intrinsic property of AK.

6.2 Future Directions

Steady-state kinetic methods, described within this thesis, have been used to identify that the presence of phosphate alters the k_{cat} , K_m and K_i values for AK. Mutational analysis has identified a region distal to the active site which may be exploited in the creation of novel pharmaceutical agents. The most important question remaining is which step in the AK reaction pathway is affected by phosphate. Studies such as solvent viscosity and isotope effects could be employed to determine if substrate binding or product release and/or proton abstraction, respectively, is limiting. If an effect is seen, these studies would be repeated in the presence of phosphate.

Transient-state kinetic analysis, as reviewed by Johnson (1992) [8], allows for the direct measurement of the sequential events leading to the conversion of substrates through to products. These studies may provide insights as to the specific role of phosphate on the activity of AK. Stopped-flow or substrate trapping methods would measure the rates of substrate binding and release [8]. Presteady-state product formation measured by chemical-quench-flow techniques would provide information regarding the rate of the chemistry step of the reaction and if events immediately after the bond-breaking or bond-formation steps are involved in the limitation of overall activity [8]. Chemical-quench-flow measurements of single enzyme turnover can also yield information regarding enzyme intermediates [8]. These analyses would be performed both in the presence and absence of phosphate.

AK inhibitors which include substituents which bind at the NXXE motif should also be investigated. During an hypoxic episode in mammalian tissues, the concentration

receptors which prevent tissue damage. Substrate inhibition of AK at these high concentrations of adenosine may be an endogenous response to augment this protective effect. If phosphate, or phosphorylated metabolites can stimulate the activity of AK, this increased concentration of adenosine would rapidly be converted to AMP, thereby decreasing the concentration of adenosine available for interaction with cell surface receptors.

Therefore, pharmaceuticals designed to inhibit AK activity which have incorporated substituents to bind at the NXXE motif may result in increased specificity of binding and decrease the likelihood of being displaced by phosphorylated metabolites. A hypothetical inhibitor 5'-amino-pentyl-5'-deoxy adenosine phosphate has been created with HyperChem, version 4, and modeled in to the active site of CHO AK with SwissProt PDB viewer (Fig. 6.1). The residue interactions of the adenosine moiety found in the human AK structure is retained in this model. New interactions include H-bonding of the phosphate group with Asn 239, Glu 242, Asn 212, and Ser 214. Hydrogen bonds are shown in green (within 3.5 angstroms), and close interactions of residues to substrate are shown in pink (3.5 to 4 angstroms). The hydrophobic interaction of Phe 186 with the adenine base is shown as a pink cloud.

Inhibitors such as the modeled 5'-amino-pentyl-5'-deoxy adenosine phosphate, and theoretical 4-amino-5-iodo-7-(5'-deoxy-5'-amino-pentyl- β -D-ribofuranosyl) phosphate, should be synthesized and analyzed for their ability to inhibit AK activity. These compounds may be more specific for AK with reduced non-specific binding to cell surface adenosine receptors.

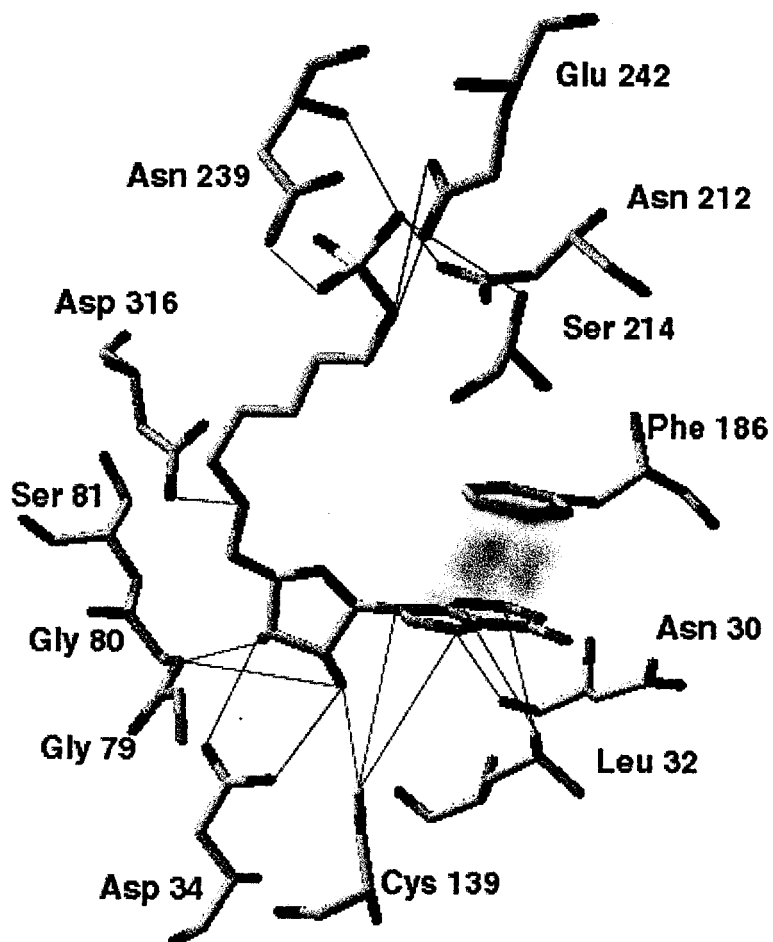


Figure 6.1 The Adenosine Binding site of AK.

The residues at the adenosine binding site which interact with adenosine and a putative inhibitor are shown. Based on the coordinates for human AK bound with adenosine, inhibitor 5'-amino-pentyl-5'-deoxy adenosine phosphate was modeled into the active site.

6.3 References

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