

# ADENYLATE KINASE

The various forms and their role in cellular energy homeostasis

ROHAN SINGH

Exam Roll No: 21025746024

KENANANDA LAIRENJAM

Exam Roll No: 21036746028

SOUMYADEEP BAIDYA

Exam Roll No: 21056746043

## ABSTRACT

Adenine nucleotides are vital for energy and metabolism on cellular level. They are involved in energy production and transfer, metabolic signaling, DNA synthesis, coenzyme function, cellular motility, genetic and hormonal expressions. Adenylate kinase (AK) are ubiquitous enzymes found in almost all living organisms with high similarity across species. It catalyzes the reversible transfer of phosphate group between adenine nucleotides, mainly AMP and ATP. It is the only enzyme within the cell that inter converts adenine nucleotides to regulate their levels in various cellular components, which is crucial for energy metabolism, signaling and overall homeostasis of the cell. So far, nine human AK isoforms have been identified which differ in their location within the cell, distribution across various tissues, specificity towards substrate and phosphate donor, kinetics, physiological functions and pharmacological implications. AK family plays a crucial role in processes such as mono and diphosphate kinases, purine nucleotide synthesis, intracellular AMP-ATP shuttle and activation of anti-cancer drugs such as Nucleoside analogues which depend on intracellular phosphorylation. Various assays have been developed over the years to determine the factors affecting AK activity. *This paper provides a rich review of the structure, mechanism of action, isoforms, various activity assays, physiological functions and recent research on AK such as Molecular Dynamics simulations for energetics and structural analysis. Additionally, it examines the impact of dysfunction in AK activity and how that leads to severe medical conditions such as cancer, neurodegenerative disorders, and heart diseases.*

UNIVERSITY OF DELHI

PROJECT WORK

April 26, 2023





DEPARTMENT OF CHEMISTRY  
UNIVERSITY OF DELHI  
DELHI-110007

### CERTIFICATE

This is to certify that this project entitled "**Adenylate Kinase: Review of Structure, Mechanism and Functions of AK isoenzymes**" has been compiled by **Rohan Singh** (Examination Roll No: 21025746024) , **Kenananda Laienjam** (Examination Roll No: 21036746028) and **Soumyadeep Baidya** (Examination Roll No: 21056746043), students of **M.Sc. Physical Chemistry, Group 1, 4th semester (Paper no. 4312)** purely on the basis of literature survey towards the partial fulfilment of the requirements for the award of degree of Master of Science (M.Sc.) in chemistry of the University of Delhi.

**Prof. Subho Mozumdar**

Project Supervisor  
Department of Chemistry  
University of Delhi

**Prof. S.K Awasthi**

Head of Department  
Department of Chemistry  
University of Delhi

## DECLARATION

The project entitled “**Adenylate Kinase: Review of Structure, Mechanism and Functions of AK isoenzymes**” is in partial fulfilment of the requirements for the award of Degree of Master of Science (M.Sc.) in Chemistry of the University of Delhi.

We hereby state that:

- The project is in original script and has been written in our own language.
- Content of this project does not contain any text, diagram without appropriate acknowledgement.
- The work hasn't been submitted earlier for any diploma and/or degree of the University of Delhi or any other University.

We stand solely responsible for the errors/ mistakes that might have occurred in the compilation of the project report despite all precisions taken to the best of our ability.

**ROHAN SINGH**

Exam Roll No: 21025746024

**KENANANDA LAIRENJAM**

Exam Roll No: 21036746028

**SOUMYADEEP BAIDYA**

Exam Roll No: 21056746043

M.Sc. Physical Chemistry

Group - I, Semester - IV

Department of Chemistry

University of Delhi

## ACKNOWLEDGEMENT

We would like to thank our supervisor, **Prof. Subho Mozumdar** (Professor, Department of Chemistry, University of Delhi) for providing us an opportunity to work on this project. His constant guidance and sustained interest to attain the objectives are gratefully acknowledged. His support and faith in us have boosted our confidence in learning new things which led to the successful completion of this project.

We would also appreciate faculty and Department of Chemistry for providing the work culture and environment.

Special thanks to Central Science Library (CSL) for giving us access to read and download research articles. We extend our gratitude to all teachers, friends and family for their invaluable support.

### **ROHAN SINGH**

Exam Roll No: 21025746024

### **KENANANDA LAIRENJAM**

Exam Roll No: 21036746028

### **SOUMYADEEP BAIDYA**

Exam Roll No: 21056746043

M.Sc. Physical Chemistry

Group - I, Semester - IV

Department of Chemistry

University of Delhi

# Contents

<b>I</b>	<b>Introduction</b>	<b>5</b>
<b>II</b>	<b>Structure of Adenylate Kinase</b>	<b>6</b>
II.i	Domains and Binding Sites . . . . .	7
II.ii	Predominant Conformations . . . . .	7
<b>III</b>	<b>Mechanism of Action of AK</b>	<b>9</b>
III.i	Conformational Mechanism . . . . .	9
III.ii	Inhibition Testing . . . . .	10
III.iii	Molecular Dynamics Simulation . . . . .	11
<b>IV</b>	<b>Enzyme Assay</b>	<b>14</b>
IV.i	Introduction . . . . .	14
IV.ii	Conventional AK Assays . . . . .	14
IV.ii.1	Conventional: Forward Reaction . . . . .	15
IV.ii.2	Conventional: Backward Reaction . . . . .	15
IV.ii.3	Limitations of Conventional Assays . . . . .	16
IV.iii	Assays based on pH . . . . .	16
IV.iii.1	Principle . . . . .	17
IV.iii.2	Inspiration from other enzyme assays . . . . .	17
IV.iv	AK assay based on pH indicator . . . . .	17
IV.iv.1	Principle . . . . .	17
IV.iv.2	Sensitivity of Bromothymol Blue . . . . .	19
IV.iv.3	Effect of AK content . . . . .	20
IV.iv.4	Effect of Temperature and KCl on AK activity . . . . .	21
<b>V</b>	<b>Isoforms of Adenylate Kinase</b>	<b>22</b>
V.i	Introduction . . . . .	22
V.ii	General differences in Human AK Isoforms . . . . .	22
V.iii	A deeper view of Human AK isoforms . . . . .	25
V.iii.1	Adenylate Kinase 1 (AK1) . . . . .	25
V.iii.2	Adenylate Kinase 2 (AK2) . . . . .	28
V.iii.3	Adenylate Kinase 3 (AK3) . . . . .	29

V.iii.4	Adenylate Kinase 4 (AK4) . . . . .	29
V.iii.5	Adenylate Kinase 5 (AK5) . . . . .	30
V.iii.6	Adenylate Kinase 6 (AK6) . . . . .	30
V.iii.7	Adenylate Kinase 7 (AK7) . . . . .	31
V.iii.8	Adenylate Kinase 8 (AK8) . . . . .	32
V.iii.9	Adenylate Kinase 9 (AK9) . . . . .	32
<b>VI Functions of Adenylate Kinase</b>		<b>33</b>
VI.i	AK Shuttle . . . . .	33
VI.ii	Nucleoside Diphosphate Kinases (NDPKs) . . . . .	35
VI.ii.1	Introduction and Mechanism . . . . .	35
VI.ii.2	Adenylate Kinase as NDPKs . . . . .	36
VI.iii	Intracellular Synthesis of Purine Nucleotides . . . . .	37
VI.iii.1	Importance of Nucleotides . . . . .	37
VI.iii.2	The <i>de-novo</i> pathway . . . . .	38
VI.iii.3	The <i>Salvage</i> Pathway . . . . .	39
VI.iv	Nucleoside Analogues . . . . .	40
VI.iv.1	Introduction, Mechanism and Effect . . . . .	40
VI.iv.2	Examples . . . . .	42
<b>VII Conclusion</b>		<b>43</b>

## List of Tables

1	Correlation between the absorbance of Bromothymol blue at 614 nm (A <sub>614</sub> ) with pH <sup>[12]</sup> . . . . .	19
2	Residues, Genes and Database Identifiers of human AK isoforms . . .	23
3	Subcellular location and tissue distribution of human AK isoforms <sup>[1]</sup>	24
4	Phosphate donor and substrate specificity of the human AK isoforms <sup>[1]</sup> . . . . .	25

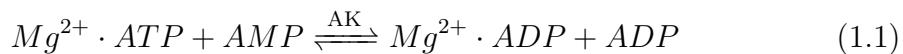
## List of Figures

1	Adenylate Kinase (AK) structure at two different stages of phosphate transfer reaction between ATP and AMP. Mg <sup>2+</sup> -ATP is bound between CORE and LID (ATP binding site), while AMP is sandwiched between CORE and NMP (AMP binding site). ATP, ADP and AMP are represented by ball and stick models [5] . . . . .	8
2	Generic catalytic cycle of Adenylate Kinase (AK). E represents the enzyme AK, while AMP, ADP and ATP are adenosine nucleotides. Ternary complex represents AK bound to two ligands simultaneously	10
3	Key residues in Adenylate Kinase of <i>Escherichia coli</i> crystallized with AP5A inhibitor . . . . .	11
4	RMSD (root mean square deviation) values calculated for each domain of AK in the open and closed conformations from MD simulation . . . . .	12
5	MD simulation of AK conformations . . . . .	13
6	Spectrophotometric assay for AK activity using Bromothymol blue as pH indicator [12] . . . . .	18
7	Sensitivity of Bromothymol blue towards H <sup>+</sup> ion concentration [12] . .	19
8	Effect of AK content on spectrophotometric assay [12] . . . . .	20
9	Effect of temperature and KCl on the activity of AK [12] . . . . .	21
10	Sequence, crystal structure and genetic tree of human AK isoforms. Red, yellow and green colored areas represents CORE, LID and NMP domains of AK respectively [1] . . . . .	26
11	Cytosolic and mitochondrial AKs with chromosomes containing their respective genes (only exception is the gene of cytosolic AK7, present on chromosome 14) [4]. Abbreviations $\Rightarrow$ C: Cytosol, M: Mitochondria, chr: Chromosome . . . . .	33
12	Transfer of high energy $\beta$ and $\gamma$ phosphoryls of ATP from production to consumption site via ATP shuttle. (i.m: inner membrane, o.m outer membrane) [44] . . . . .	34

13	Ping-Pong mechanism followed by NDPKs for the phosphorylation of NDP or dNDP substrate. This example use ATP as phosphate donor [46]	36
14	The <i>de-novo</i> synthesis of purine nucleotides inside a mammalian cell <sup>[1]</sup> .	39
15	Nucleoside analogues	43

# I Introduction

Adenylate Kinase (ADK or AK, more commonly known as Myokinase) is a ubiquitous and prolific enzyme, present in almost all eukaryotic and prokaryotic cells. It belongs to a family of kinase enzymes, which are specific type of phosphotransferase that catalyze the transfer of phosphate group from a high energy donor to an acceptor substrate in a process called *phosphorylation*. In case of kinase catalyzed reactions, high energy phosphate donor is usually Adenosine Triphosphate (ATP). The reversible reaction catalyzed by Adenylate Kinase is [1]:



where AMP and ADP are Adenosine Monophosphate and Adenosine Diphosphate respectively.

The systematic name of the most common form of AK (owing to the reaction catalyzed) is *ATP:AMP phosphotransferase*, with an enzymatic identifier of *EC 2.7.4.3*. As such, Adenylate Kinase catalyzes the inter-conversion of various adenine nucleotides (AMP, ADP and ATP) which are the main components of cellular energy. Thus, AK plays a crucial role in regulating and balancing the production and expenditure of cellular energy, by constantly monitoring the levels of ATP, ADP and AMP inside the cell. It is important for achieving energy homeostasis within the cell, and is involved in a wide range of endergonic physiological processes, including muscle contraction, signal transduction and active ion transport.

**Role of Mg<sup>2+</sup>:** For ATP to be able to interact with AK, a metal ion such as magnesium or manganese must first bind to it, forming a complex with high energy phosphate groups of ATP and other water molecules. These coordinated water molecules can form hydrogen-bonds with active site of the enzyme, which increases the number of interactions and facilitates the binding of ATP with the enzyme [2]. Moreover, Mg<sup>2+</sup> counterbalances excess negative charge density on ATP due to three phosphate groups, which allows for a better interaction with AMP within the enzyme pocket. In effect, *presence of Mg<sup>2+</sup> increases electrophilicity of ATP*. However, Mg<sup>2+</sup> is only bound electrostatically within the active site and dissociates

easily, which leads to the simplified form



AK in eukaryotes is mostly present in the region between inner and outer mitochondrial membranes, while it is typically found in the cytoplasm and periplasm of Gram-negative bacteria. It is necessary for life since it is the only enzyme made by cells that can phosphorylate AMP into ADP. It is a considerably stable protein with significantly long intracellular lifespan<sup>[3]</sup>. A strikingly unique feature of AK is the existence of many isoforms of the enzyme, with different properties such as molecular weight, phosphate donor and substrate selectivity, intracellular location, kinetics and physiological impact. In humans, 9 such isoforms have been identified, most of which are selective towards ATP as phosphate donor, while some can also use other Nucleoside Triphosphate (NTPs) as donors. Specificity towards AMP as substrate also varies in similar fashion.

Dysregulation in Adenylate Kinase activity has been linked to a number of illnesses, including cancer, heart failure, and neurodegenerative disorders. Deficiency or mutation of a particular isoform of AK is associated with disorders specific to that isoform, some being life threatening. As a result, therapeutic intervention targeting AK might be revolutionary to treat these conditions <sup>[4]</sup>.

Overall, AK is necessary for regular physiological function and plays a crucial part in preserving cellular energy metabolism. For a deeper comprehension of AKs function in cellular processes and the creation of innovative therapeutic approaches, additional focus on AKs structure, mechanism of action, isoforms, physiological function and effects is required.

## II Structure of Adenylate Kinase

Adenylate kinase (AK) in humans is composed of 194 amino acid residues with a molecular weight of approximately 22 kDa. The bacterial AK is usually longer than its eukaryotic counterpart and is made up of approximately 210-220 amino acids. The protein sequence of AK is highly conserved across species, with over 90% sequence identity between human and mouse AK.

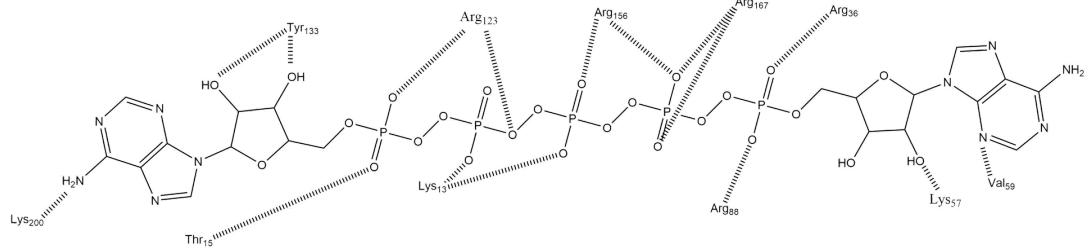
## II.i Domains and Binding Sites

The crystal structure of human AK has been determined by X-ray crystallography, revealing a compact globular protein composed of a *three domains, namely the CORE, LID and Nucleoside Mono-Phosphate (NMP) substrate binding region*. Overall, AK features two binding sites, one for each of phosphate donor and acceptor substrate. CORE domain contains glycine rich region where phosphate donor binds, called P-loop [1]. It is highly conserved across species, while LID and NMP constitute variable surface loops. The CORE domain of human AK consists of six  $\beta$ -strands and six  $\alpha$ -helices, forming a central  $\beta$ -sheet flanked by  $\alpha$ -helices on either side. The surface loop region (LID and NMP) of human AK connects the  $\beta$ -strands and  $\alpha$ -helices of the CORE domain and contains the ATP and AMP binding sites. The CORE domain is conformationally unaffected during the enzymatic action of AK, while LID and NMP domains (surface loop region) are highly flexible and undergoes significant conformational changes upon substrate binding, triggering the catalytic activity of the enzyme.

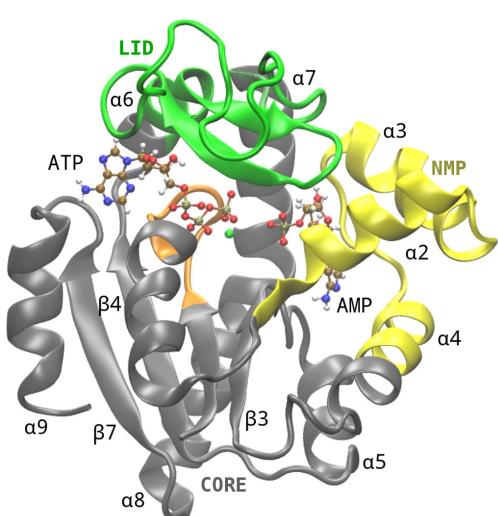
The ATP and AMP binding sites of human AK are located on opposite sides of the enzyme, with the ***ATP site situated between CORE and LID domains*** (near N-terminus of the protein) and the ***AMP site located between CORE and NMP*** (near C-terminus of the protein). The ATP site of human AK is composed of residues from the  $\beta$ 1- $\beta$ 2 loop, the  $\beta$ 4 strand, and the  $\alpha$ 4 helix, while the AMP site is composed of residues from the  $\beta$ 5 –  $\beta$ 6 loop and the  $\alpha$ 5 helix. This is illustrated in figure 1.

## II.ii Predominant Conformations

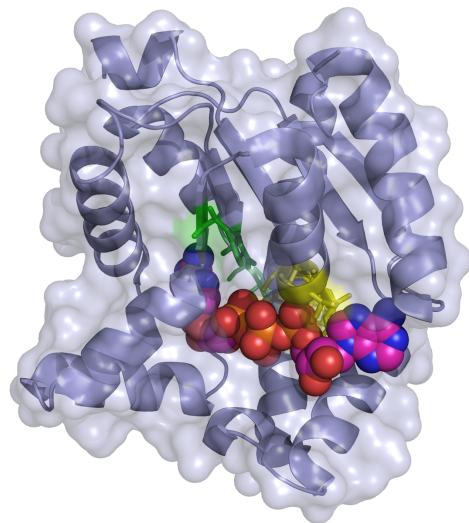
AK exists in a series of conformations as the phosphorylation reaction proceeds. Three main conformations are normal, open and closed. Flexibility and plasticity allow enzyme to adopt various conformations, and bind to substrates, form oligomers, aggregate, and perform mechanical work. Large conformational changes in proteins play an important role in enzymatic activity and cellular signaling. Adenylate Kinase is a signal transducing protein, for which the balance between conformations regulates activity. AK has a locally unfolded



(a) Residues of AK in *Escherichia coli* involved in substrate binding



(b) AK bound to ATP and AMP. The three domains CORE (residues 1–29, 68–117, 161–214), LID (residues 118–160) and NMP (residues 30–67) are colored gray, green and yellow respectively. Glycine rich ATP binding P-loop (residues 7–13) within CORE is colored orange



(c) 3D Ribbon model of AK complexed with 2 ADP molecules. The active center and ATP binding site are colored yellow and green respectively

Figure 1: Adenylate Kinase (AK) structure at two different stages of phosphate transfer reaction between ATP and AMP.  $Mg^{2+}$ -ATP is bound between CORE and LID (ATP binding site), while AMP is sandwiched between CORE and NMP (AMP binding site). ATP, ADP and AMP are represented by ball and stick models [5]

state that becomes depopulated upon binding. Localized regions of a protein unfold as a result of competing strain energies during conformational transitions, which relaxes strain and increases efficiency of catalysis.

The local thermodynamic stability of the substrate-binding domains  $LID_{ATP}$  and  $NMP_{AMP}$  has been shown to be significantly lower when compared with the CORE domain in the absence of substrates. It has also been shown that  $LID_{ATP}$  and  $NMP_{AMP}$  can fold and unfold in a "non-cooperative manner" [6]. Binding of the substrates causes preference for *closed* conformations amongst those acquired by AK. These closed conformations discourage water from entering the active site and prevent wasteful hydrolysis of AMP and ATP in addition to helping optimize alignment of substrates for phosphoryl-transfer [7].

Furthermore, apoenzyme is shown to predominantly exists in closed conformations of the  $LID_{ATP}$  and  $NMP_{AMP}$  domains, even in the absence of substrates. As the transfer of phosphate completes, Adenylate Kinase adopts an *open* conformation that enables product (two ADP molecules) to detach (figure 1c). Conformations of AK with regard to its catalytic action are further explored in section III.

## III Mechanism of Action of AK

### III.i Conformational Mechanism

The mechanism of action of Adenylate Kinase involves a series of conformational changes that are triggered upon binding of phosphate donor and substrate, which facilitates the transfer of phosphate group from donor to substrate.

In the absence of any bound ligand (donor or substrate), the enzyme is in the unligated "open" conformation. As  $Mg^{2+}$  complexed phosphate donor (ATP or other) binds with the enzyme, it triggers a conformational change that causes open surface loop of LID domain to close. This in turn signals NMP binding site to close with substrate bound within the pocket. As a result, AK adopts "closed" conformation [1].

In closed state, AK globular structure shrinks, causing water molecules to expel from the enzymatic cavity, which brings the substrate in proximity with phosphate

donor. In addition, the presence of  $Mg^{2+}$  increases electrophilic character of ATP. Combined effect of both these factors results in the decrease of energy barrier for the nucleophilic attack on the  $\gamma$ -phosphoryl of ATP by  $\alpha$ -phosphoryl of AMP. This causes  $\gamma$ -phosphate to migrate from ATP to AMP, resulting in the formation of two ADP molecules.

Phosphate transfer triggers further conformation change in AK which causes LID domain to open up, releasing one ADP molecule, which was bound to phosphate donor binding site. The opening of LID domain triggers another conformation change which signals the substrate binding NMP domain to open up and release the second ADP molecule. This marks the completion of one catalytic cycle, resulting in AK back to its open conformation, free to catalyze another cycle. Catalytic cycle of AK is illustrated in figure 2.

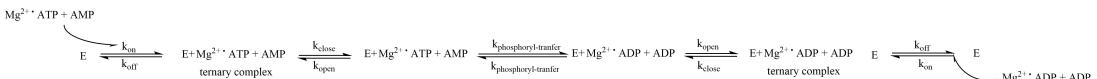


Figure 2: Generic catalytic cycle of Adenylate Kinase (AK).  $E$  represents the enzyme AK, while AMP, ADP and ATP are adenosine nucleotides. Ternary complex represents AK bound to two ligands simultaneously

### III.ii Inhibition Testing

Crystal structure of Adenylate Kinase from *Escherichia coli* with AP5A (P1,P5-Di(adenosine-5') pentaphosphate) inhibitor shows that AP5A binds with Arg88 at  $\alpha$  phosphate group. This inhibition results in 99% loss of catalytic activity of AK, which suggest that Arg88 plays a crucial role in the phosphoryl transfer reaction [8] (figure 3). Another residue in CORE domain of AK that remains highly conserved across species is Arg119, located at adenosine binding region of AK and binds with purine base (Adenine) within the active site. Since it has been established that some isoenzymes of AK can accept nucleoside triphosphates (NTPs) other than ATP as phosphate donors, this non-specificity of AK regarding ATP can be explained based on relatively insignificant interaction of purine base with Arg119 in donor binding pocket of AK.

Other major conserved residues in AK (from *Escherichia coli*) are Lys13,

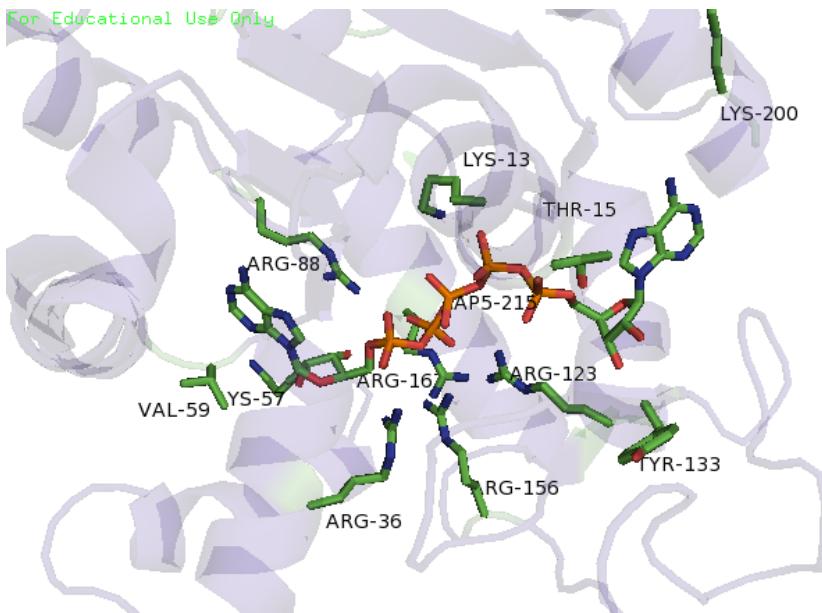


Figure 3: Key residues in Adenylate Kinase of *Escherichia coli* crystallized with AP5A inhibitor

Arg123, Arg156, and Arg167 which forms a network of positive amino acids. This conserved positive network of residues stabilize the accumulation of negative charge on the phosphoryl group during the transfer. This lysine-arginine network is also bound by two distal aspartate residues, that causes the enzyme to fold and lose some of its flexibility.

### III.iii Molecular Dynamics Simulation

One way to actually verify how conformation changes are the key to catalytic activity of AK, and how CORE domain is conformationally stable, while LID and NMP domains are the most conformationally affected is using Molecular Dynamics (MD) simulations [5]. Conformation sampling of AK can be done using about  $1\mu\text{s}$  of metadynamics sampling rate, which allows for the collective motion of X-ray structural functions of AK to be simulated in  $1\mu\text{s}-\text{ms}$  timescale. This kind of metadynamics simulations reveals that the major conformational changes occur at the LID and the NMP domains, while CORE domain is relatively unaffected.

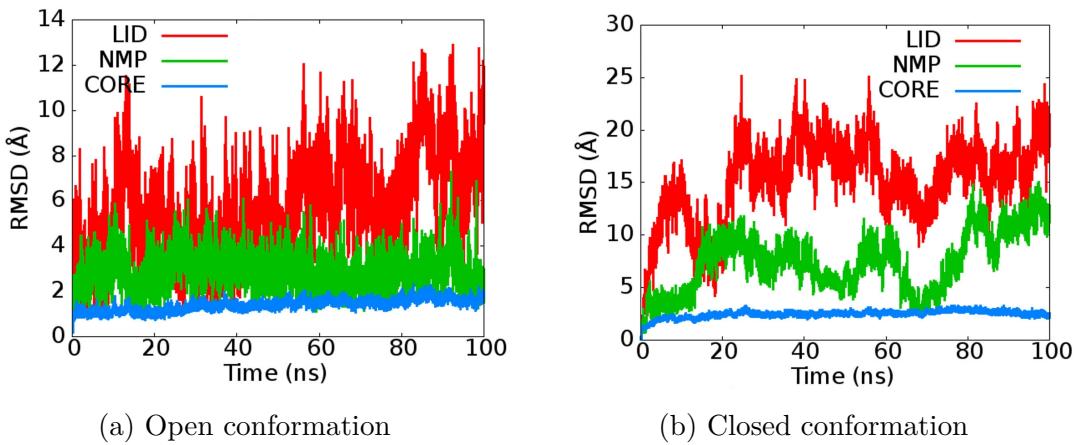
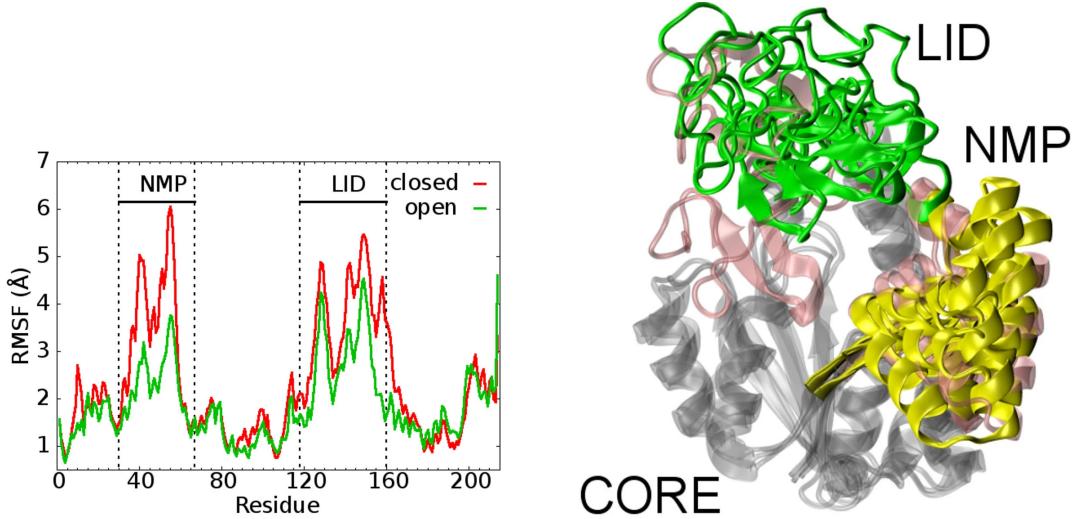


Figure 4: RMSD (root mean square deviation) values calculated for each domain of AK in the open and closed conformations from MD simulation

From the MD simulation of X-ray structure of AK, it is evident that the enzyme adopts several conformations, separated by only a few  $k_B T$  (Thermal energy at simulation temperature). These conformations can be interpreted relative to open and closed conformations of AK. Plots of **RMSD** (Root Mean Square Deviation, represents average distance of separation between a group of atoms in a particular conformation) values with time in nano scale for open and closed conformations are illustrated in figure 4. These plots describes how CORE, LID and NMP domains in open and closed states of AK evolve over time. Another plot of **RMSF** (Root Mean Square Fluctuations, measure of average deviation of a group of atoms from their respective mean positions over time) of backbone amino acid residues in open and closed conformations is given in figure 5a. Superimposition of some selected MD simulation conformations with the open and closed X-ray states of AK is illustrated in figure 5b, which shows how well simulated conformations correlate with actual states of AK crystallographic structure.

From figure 4 and 5a, it is evident that the CORE domain is conformationally stable, while the LID domain transverses a wider conformational space than that of NMP. LID domain is more flexible than the NMP domain, and attains several conformations ranging from the crystallographic open to the closed structure and many conformations in



(a) RMSF (root mean square fluctuation) of the backbone atoms calculated from MD simulation for the open and closed AK conformations

(b) Selected MD simulation conformations superimposed over the open and closed X-ray states of AK. The X-ray conformations are represented in pink

Figure 5: MD simulation of AK conformations

between. Amongst several of these possible conformations, open and closed states bear lowest free energy and are the most stable, implying these are the most prominent conformations of AK. Furthermore, open conformation is 1-2  $k_B T$  lower in free energy than the closed state, which seems logical given the fact that the open state corresponds to binding of ligands to AK.

Thus, MD simulation of crystallographic AK suggest that as the LID and NMP domains open up, the resulting conformation of enzyme favors donor and substrate binding. When the enzyme adopts a closed LID-NMP conformation, it locks bound ligand in place facilitating phosphoryl transfer. After reaction completes, LID and NMP open up yet again to attain a conformation suitable for releasing the product. These results confirms to previous observations. More detailed information on the specific conformations assumed by the LID and NMP domains can be obtained from the Free Energy Surfaces (FES) computed as a function of Collective Variables (CVs).

## IV Enzyme Assay

### IV.i Introduction

Enzymatic assay is a laboratory procedure to measure the activity of an enzyme, and the factors affecting it. These assays are widely used in the field of enzymatic kinetics and inhibition.

**Enzyme Activity (EA):** It is defined as the number of moles of substrate converted by the enzyme present, per unit time. For a simple enzyme catalyzed reaction  $S \xrightarrow{E} P$ , the rate of product formation  $r$  will be given as

$$r = \frac{d[P]}{dt} = -\frac{d[S]}{dt} \quad (4.1)$$

where  $[S]$  and  $[P]$  are the molar concentrations of substrate and product in moles/L respectively. For a given enzyme concentration, rate of the reaction increases as the substrate concentration is increased. However, after a certain substrate concentration, rate of the reaction balances off due to the limited amount of enzymatic active sites present.

If  $N_S$  denotes the number of moles of the substrate, and  $V$  be the reaction volume, then  $[S] = N_S/V$ . Using equation (4.1), enzyme activity  $EA$  can be expressed as

$$EA = -\frac{dN_S}{dt} = r \times V \quad (4.2)$$

The SI unit of EA is *katal* (1 katal = 1 mol/s). However, katal is too large from any practical viewpoint. Hence, more commonly used is the *enzyme unit* U (1 U = 1  $\mu\text{mol}/\text{min}$  = 16.67 nano katal).

### IV.ii Conventional AK Assays

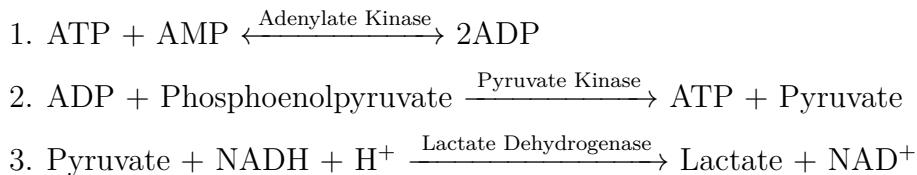
All of the AK assays proposed so far measure AK activity by tracking changes in some characteristic detectable property, as the phosphoryl transfer reaction catalyzed by AK proceeds. These include changes in the absorption of light, pH of the reaction medium or the concentration of product of coupled reaction.

For the AK catalyzed inter-conversion of AMP, ADP and ATP as described by equation (1.2), the forward reaction produces two ADP molecules using ATP

and AMP, while the backward reaction produces one molecule each of ATP and AMP using two ADP molecules. Conventional assays for the determination of AK activity for both forward and reverse direction are as follows

#### IV.ii.1 Conventional: Forward Reaction

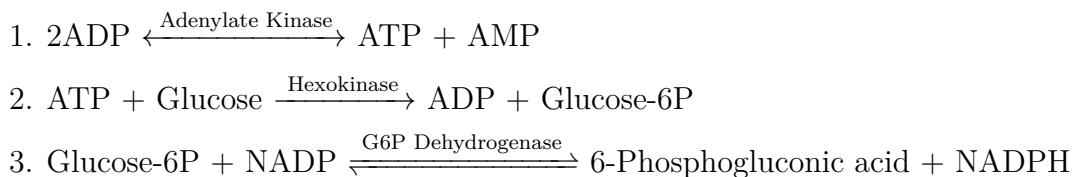
Activity of AK for the forward reaction (i.e production of two ADP molecules) can be measured by coupling this reaction with those catalyzed by pyruvate kinase and lactate dehydrogenase sequentially [9].



ADP produced from the main reaction is phosphorylated to ATP by Phosphoenolpyruvate in the presence of Pyruvate Kinase. Pyruvate produced is then used to oxidize NADH to NAD<sup>+</sup> in presence of Lactate Dehydrogenase. Since NADH strongly absorbs at 340 nm (and NAD<sup>+</sup> is virtually transparent at 340 nm), the rate of consumption of NADH in the final step can be tracked by monitoring the decrease in absorption at 340 nm with time. Assuming Phosphoenolpyruvate, NADH, Pyruvate Kinase and Lactate Dehydrogenase are all present in excess and are not rate limiting, rate of the main reaction can be given by the rate of consumption of NADH determined spectrophotometrically.

#### IV.ii.2 Conventional: Backward Reaction

Likewise, activity of AK for the backward reaction (i.e production of AMP and ATP) can be measured by coupling this reaction with those catalyzed by Hexokinase and Glucose-6-Phosphate Dehydrogenase sequentially (spectroscopic assay originally given by Oliver<sup>[10]</sup> and modified by Chiu<sup>[11]</sup>).



ATP produced from the main reaction is used to phosphorylate Glucose in the presence of Hexokinase, which yields Glucose-6-Phosphate. This in turn is used to reduce NADP to NADPH in the presence of Glucose-6-Phosphate Dehydrogenase enzyme. Like NADH, its phosphorylated form NADPH also strongly absorbs at 340 nm. Thus, the rate of production of NADPH in the final step can be tracked by monitoring the increase in absorption at 340 nm with time. Assuming Glucose, NADP, Hexokinase and Glucose-6P-Dehydrogenase are all present in excess and are not rate limiting, rate of the main reaction can be given by the rate of production of NADPH determined spectrophotometrically.

#### **IV.ii.3 Limitations of Conventional Assays**

Conventional assays for the determination of AK activity were used for several decades. However, they have some limitations listed below [12].

- Conventional assays are mostly based on coupled reactions, which are multi-step processes that require other enzymes and lot of time. Error may induce at each step making whole assay highly error prone.
- Use of other enzymes makes it difficult to study the effect of activators and inhibitors on the activity of AK.
- Initial rate of the main reaction catalyzed by AK cannot be determined using such coupled reaction assays.

These limitations calls for the development of a more accurate and convenient assay for the *in-vitro* determination of AK activity.

#### **IV.iii Assays based on pH**

pH (or acid-base) indicators are weak organic acids/bases that exhibit color change in response to changes in pH. Due to their high pH sensitivity, they are widely used to assay enzymatic reactions that proceed with change in concentration of H<sup>+</sup> ion.

#### IV.iii.1 Principle

A pH indicator mainly exist in two forms: protonated at low pH ( $\text{H-In}$ ). and deprotonated at high pH ( $\text{In}^-$ ). Each form has a characteristic color and consequently wavelength of maximum absorption ( $\lambda_{max}$ ). In a reaction mixture, both of the indicator forms coexist in a dynamic equilibrium, which is shifted either to the left or right depending on the concentration of  $\text{H}^+$  ions. Hence, absorbance of either of the two forms at their respective  $\lambda_{max}$  at a certain time instant is a measure of concentration of  $\text{H}^+$  ions within the reaction mixture at that time. The rate of production or consumption of  $\text{H}^+$  ions in the reaction is given by the rate of increase or decrease of the absorbance of either of the indicator form at its characteristic  $\lambda_{max}$ .

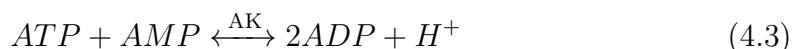
#### IV.iii.2 Inspiration from other enzyme assays

Yu [13] proposed an Arginine kinase assay based on pH indicator. Reaction catalyzed by Arginine Kinase is accompanied by the production of  $\text{H}^+$  ions, which reduces pH of the reaction mixture. Changes in pH are followed by using a mixed indicator (thymol blue + cresol red), which in its deprotonated forms strongly absorbs at 575 nm. As the reaction proceeds, pH decreases and the mixed indicator gets progressively protonated, reducing absorbance at 575 nm in accordance with Beer Lambert law. Likewise, a sensitive and rapid L-asparaginase assay using methyl red indicator was given by Dhale [14].

### IV.iv AK assay based on pH indicator

#### IV.iv.1 Principle

The forward reaction catalyzed by AK (which produces two ADP molecules using ATP and AMP) is also associated with the production of a proton ( $\text{H}^+$  ion). Thus, it can be expressed as



which indicates that the forward reaction proceeds with the decrease in pH. This fact allows for the use of an appropriate pH indicator for AK enzymatic assay.

Since AK requires  $\text{pH} \approx 7.5$  to be optimally active, it is dissolved in a pH 7.6 NaOH-glycine buffer to ensure its activity [15]. A suitable pH indicator that operates at this pH is *Bromothymol blue* (BTB), which has a pH range of 6.0-7.6. Below pH 6.0, bromothymol blue exist predominantly in protonated form with low conjugation and characteristic yellow color (Absorption  $\lambda_{max} = 427 \text{ nm}$ ). Above pH 7.6, it exist mostly in deprotonated form with significantly higher conjugation and blue color (Absorption  $\lambda_{max} = 614 \text{ nm}$ ). Between pH 6.0-7.6, it ranges from yellowish-green to bluish-green in color.

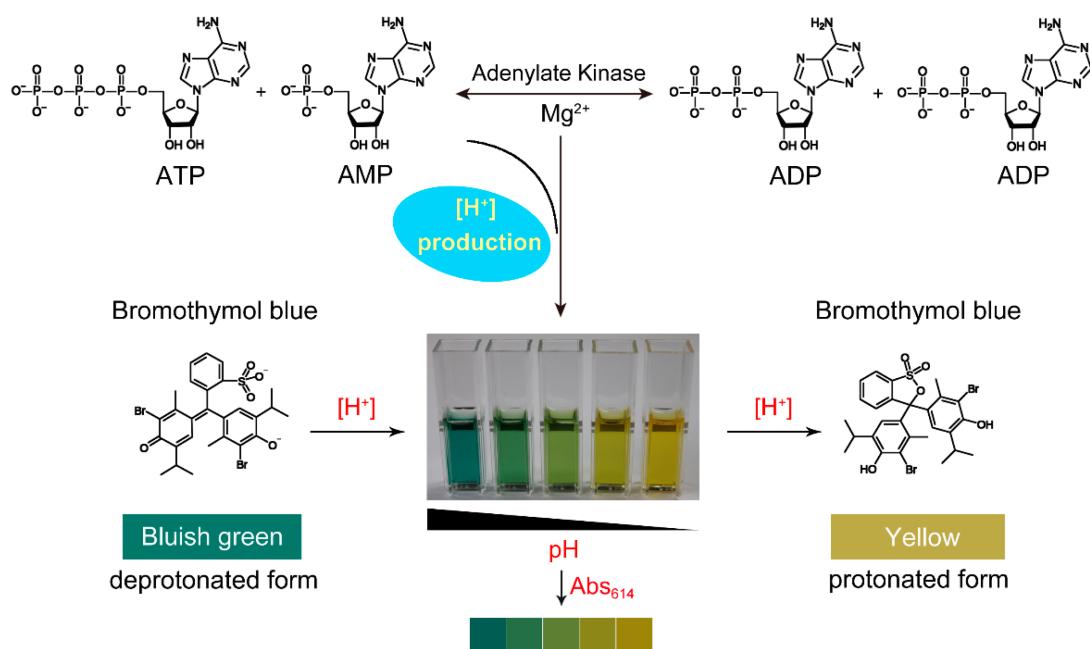
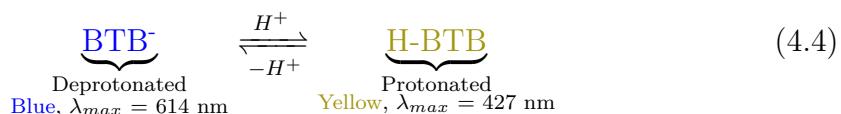


Figure 6: Spectrophotometric assay for AK activity using Bromothymol blue as pH indicator [12].

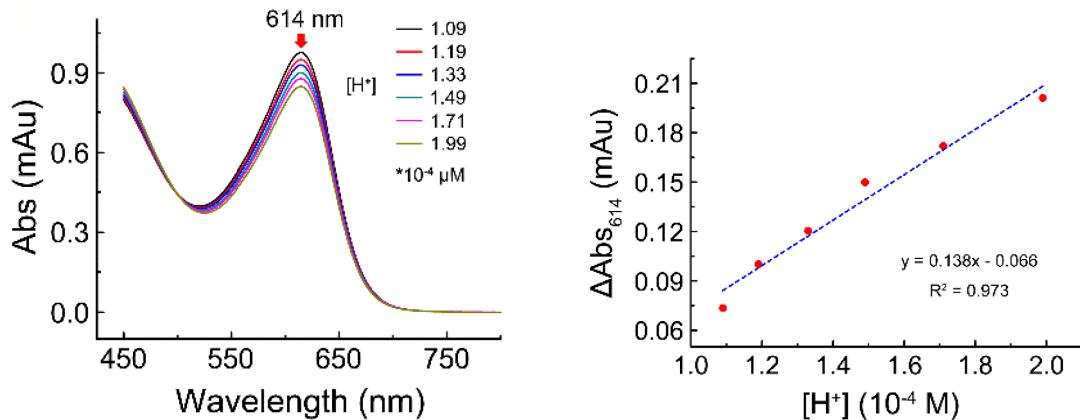
As the AK catalyzed reaction proceeds in forward direction, concentration of  $\text{H}^+$  ions increases which protonates  $\text{BTB}^-$  pushing the equilibrium in equation (4.4) to the right. This decreases absorption at 614 nm and increases at 427 nm in accordance with the Beer-Lambert Law. The rate of decrease of absorbance at 614

Table 1: Correlation between the absorbance of Bromothymol blue at 614 nm ( $A_{614}$ ) with pH [12].

pH	6.47	6.55	6.73	6.78	6.85	7.06	7.21	7.29
$A_{614}$	0.8145	0.9199	0.9438	1.0372	1.0837	1.2375	1.3650	1.4178

nm (or the rate of increase of absorbance at 427 nm) gives the rate of forward AK reaction. Hence, this method allows for the continuous and real time monitoring of AK activity in one-step using a sensitive spectrophotometer and without any coupled enzymes [12]. The principle and basic outline of this assay is illustrated in figure 6.

#### IV.iv.2 Sensitivity of Bromothymol Blue



(a) Effect of  $H^+$  ion concentration on absorption spectrum of BTB

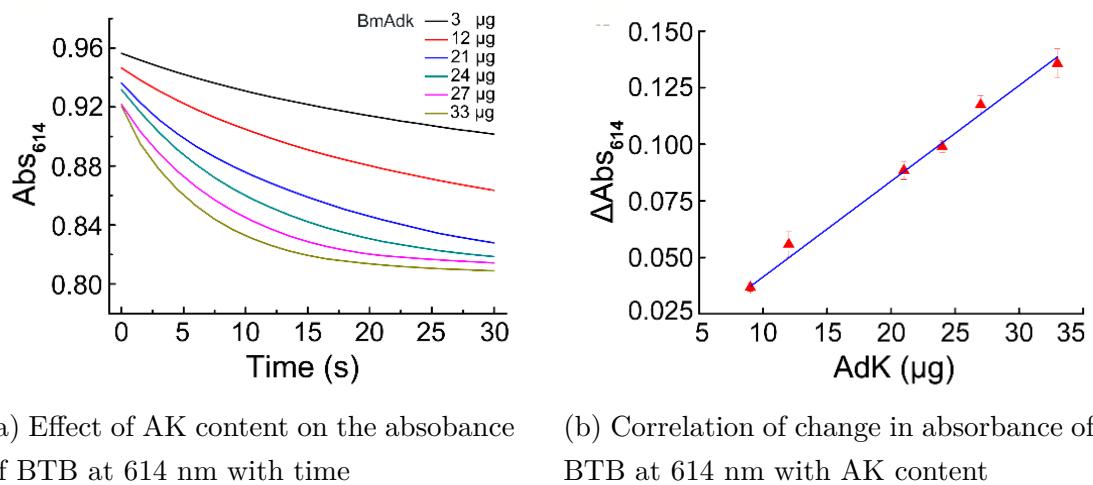
(b) Correlation of change in absorbance of BTB at  $\lambda_{max} = 614$  nm with  $H^+$  ion concentration

Figure 7: Sensitivity of Bromothymol blue towards  $H^+$  ion concentration [12].

Sensitivity of Bromothymol blue pH indicator towards AK activity assay depends on the extent to which its absorbance changes in response to change in pH (or concentration of  $H^+$  ions). For this purpose, absorption spectra of

Bromothymol blue in presence of various concentrations of HCl were recorded [12], and are illustrated in figure 7a. Irrespective of  $H^+$  ion concentration, Bromothymol blue shows absorption maxima at  $\lambda_{max} = 614$  nm. However, as  $H^+$  ion concentration in solution increases (pH decreases), absorbance of Bromothymol blue at  $\lambda_{max}$  gradually decreases. This correlation between pH and  $A_{614}$  is listed in table 1. Furthermore, change in absorbance  $\Delta A$  at 614 nm can be linearly correlated with  $H^+$  ion concentration as shown in figure 7b.

#### IV.iv.3 Effect of AK content



(a) Effect of AK content on the absorbance of BTB at 614 nm with time

(b) Correlation of change in absorbance of BTB at 614 nm with AK content

Figure 8: Effect of AK content on spectrophotometric assay [12].

As the amount of AK in the reaction mixture is increased, absorption of BTB at 614 nm ( $A_{614}$ ) decreases relatively rapidly with time. This is illustrated in figure 8a. In all the cases, decline in  $A_{614}$  is initially linear, slope of which in first few seconds gives the *initial rate of reaction*. Furthermore, changes in  $A_{614}$  of BTB are linearly correlated with the amount AK present (shown in figure 8b). This indicates that the rate of AK catalyzed reaction increases with the increase of AK content in the reaction mixture [12].

#### IV.iv.4 Effect of Temperature and KCl on AK activity

Using this spectrophotometric assay, effect of various factors on AK activity can be easily analyzed just by tracking the absorbance of Bromothymol blue (BTB) at 614 nm in the reaction mixture.

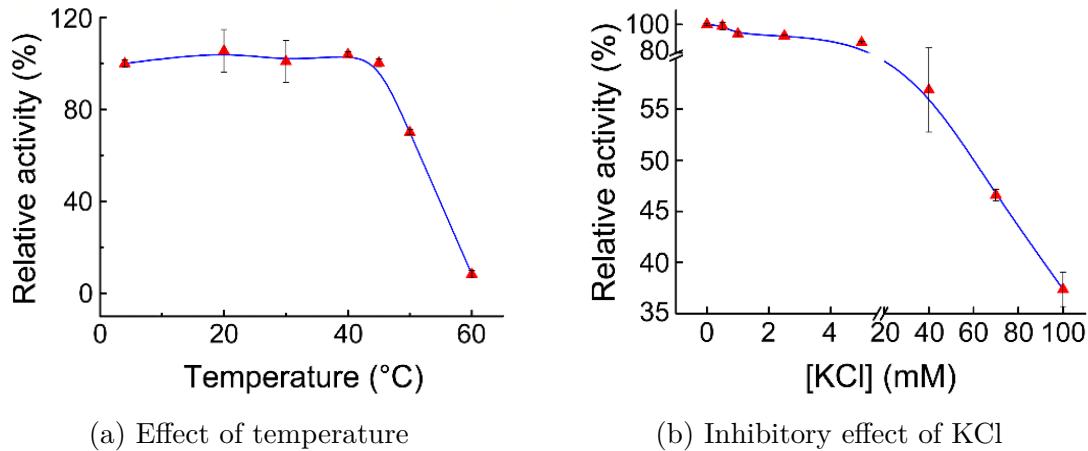


Figure 9: Effect of temperature and KCl on the activity of AK [12].

**Effect of Temperature:** As the temperature of AK reaction mixture is increased, initial rate of the reaction (given by the initial slope of  $A_{614}$  of BTB vs time as in figure 8a) remains largely unaffected upto 45°C. However, initial rate of the reaction sharply decreases after 45°C, which indicates sharp drop off in AK activity. Hence, the assay suggest that AK is thermally stable upto 45°C [12]. This is illustrated in figure 9a.

**Effect of KCl:** Likewise, as the concentration of KCl in the AK reaction mixture is increased, initial rate of the reaction (measured using spectrophotometric assay) decreases which indicates that KCl acts as an inhibitor for AK. This inhibitory effect is small at low KCl concentration (< 5mM), and becomes increasingly pronounced at higher KCl concentrations. This is illustrated in figure 9b. The assay suggest that 70 mM KCl reduces AK activity to almost 50% [12]. These observations are in accordance with those previously proved by Allan Hough [16].

## V Isoforms of Adenylate Kinase

### V.i Introduction

*Protein isoforms* (also called protein variants) represent a family of highly similar proteins, that are derived as a result of genetic mutations from a single gene or gene family. Most of the member proteins of such a family perform same or similar roles in biological system, although some isoforms may perform unique functions [17].

It has been well established that mammalian tissues contain several isoforms of AK. This feature of existence of isoforms is rare, and is specific to AK and Guanylate Kinase (GUK) among various nucleoside and nucleotide kinases. Up to this date, nine different isoenzymes of AK have been identified and characterized in human tissues. They are represented by using numerical suffixes in order of their discovery i.e AK1 to AK9. They differ in terms of subcellular location, tissue distribution, selectivity towards phosphate donor and substrate, enzymatic kinetics and therapeutic implications of dysfunction [1].

### V.ii General differences in Human AK Isoforms

Information about amino acid residues, gene encoding and identifiers of human AK isoforms from various databases is listed in Table 2. Differences in tissue distribution and intracellular location of the human AK isoforms are tabulated in Table 3, while their specificity towards phosphate donor and substrate is given in Table 4 [1, 4].

Main differences and key features of human AK isoenzymes are summarized below

1. **Substrate and Phosphate donor specificity:** Most of the isoforms of AK prefer ATP and AMP as phosphate donor and substrate respectively, but some of the isoforms can use other phosphate donors and phosphorylate other substrates. Thus, some will only use ATP, whereas others will accept GTP (Guanosine triphosphate), UTP (Uridine triphosphate), and CTP (Cytidine triphosphate) as the phosphate donors. Some of these isoforms

Table 2: Residues, Genes and Database Identifiers of human AK isoforms

Sources: [National Center for Biotechnology Information \(NCBI\)](#), [UniProt](#), [Protein Data Bank \(PDB\)](#)

AK	Residues	Gene			Accession Number
		Chromosome	Location	NCBI Gene ID	
AK1	194	9	9q34.11	203	AAH01116
AK2	239	1	1p35.1	204	AAC52061
AK3	227	9	9p24.1	50808	AAH13771
AK4	223	1	1p31.3	205	P27144
AK5	537	1	1p31.1	26289	AAH33896
AK6	536	5	5q13.2	102157402	AAO16520
AK7	723	14	14q32.3	122481	NP_689540
AK8	479	9	9q34.13	158067	NP_689785
AK9 (1)	1911	6	6q21	221264	NP_001138600
AK9 (2)	421	6	6q21	221264	NP_659462

prefer other nucleoside triphosphates entirely. For example, mitochondrial AK3 (GTP:AMP phosphotransferase) is also specific for the phosphorylation of AMP, but can only use GTP or ITP as the phosphoryl donor.

2. **Subcellular location and tissue distribution:** Some of the human AK isoforms are abundant throughout the body and are present in all tissues, while some are only present in specific tissues. Intracellular location also varies across these isoforms in a similar fashion. Isoenzymes AK1, AK5, AK7 and AK8 are all present in cytosol within the cell (*cytosolic AKs*). However, they differ in tissue distribution: AK5 is only present in the brain, AK7 is present in skeletal muscles where AK8 is absent. AK2, AK3, and AK4 are *mitochondrial AKs*. However, AK2 is found in intermembrane space of mitochondria whereas AK3 and AK4 are present in mitochondrial matrix instead. AK6 is present in nucleus, while AK9 can freely diffuse between nucleus and cytosol [18].

Table 3: Subcellular location and tissue distribution of human AK isoforms [1]

AK	Subcellular location	Tissue distribution
AK1	Cytosol	All tissues
AK2	Mitochondria (intermembrane space)	Liver, heart, skeletal muscle, kidney, lung
AK3	Mitochondria (matrix)	Liver, heart, skeletal muscle, kidney
AK4	Mitochondria (matrix)	Liver, heart, brain, kidney
AK5	Cytosol/nucleus	Brain
AK6	Nucleus	All tissues
AK7	Cytosol	Lung, trachea, testis, mammary gland, skeletal muscle
AK8	Cytosol	Liver, pancreas, lung, trachea, testis
AK9	Cytosol/nucleus	Pituitary gland, trachea, thymus, testis, mammary gland, brain, pharynx, uterus, spleen, lymph nodes

- 3. Binding and Kinetics:** In addition to the variations in intracellular location and tissue distribution of the various AK isoforms, the binding of substrate to the enzyme and kinetics of the phosphoryl transfer reaction are also quite different. For example, AK1, the most abundant cytosolic AK isoform, has a Michaelis–Menten constant ( $K_m$ , measure of the amount of substrate necessary for the enzyme to function at half its maximum velocity) of about a thousand times higher than that of AK7 and AK8, which suggests a much weaker binding of AK1 to AMP compared to AK7 and AK8 [19].
- 4. Physiological impact:** Mutation, deficiency or any other dysfunction of AK activity results in severe therapeutic implications specific to the isoform in question. For example, deficiency of AK1 results in a rare condition associated with haemolytic anaemia, while its mutation can cause language retardation, psychomotor impairment and limited learning abilities. On the other hand, deficiency and mutation of mitochondrial AK2 can cause reticular dysgenesis,

a severe immunodeficiency.

Table 4: Phosphate donor and substrate specificity of the human AK isoforms [1]

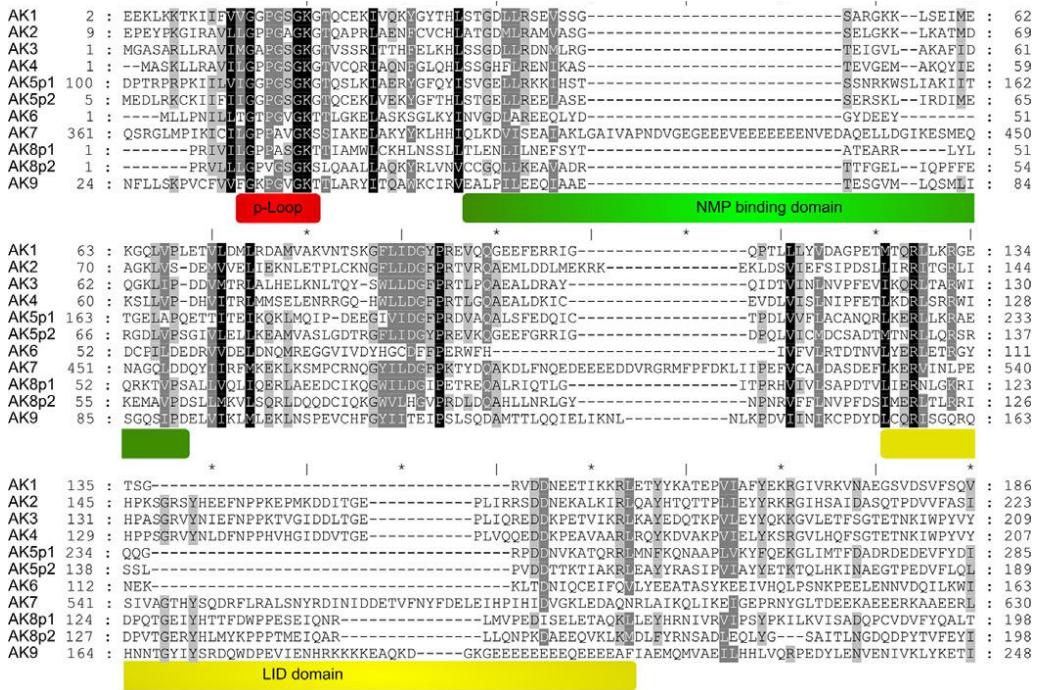
AK	Phosphate donor	Substrate
AK1	All NTPs	AMP, dAMP
AK2	All NTPs	AMP
AK3	GTP, ITP	AMP
AK4	ATP, GTP	AMP, dAMP, CMP, dCMP
AK5	ATP, GTP	AMP, dAMP, CMP, dCMP
AK6	All NTPs and dNTPs	AMP, dAMP, CMP, dCMP
AK7	ATP	AMP, dAMP, CMP, dCMP
	GTP	AMP, CMP, dCMP
AK8	ATP	AMP, dAMP, CMP, dCMP
	GTP	AMP, CMP, dCMP
AK9	ATP, GTP	AMP, dAMP, CMP, dCMP

Some residues that are essential for catalysis are highly conserved across AK isoforms. One example is the Arg residue present in the most conserved area, modification of which inactivates the enzyme. Asp residue present in the catalytic cleft of the enzyme is another such essential residue that participates in the formation of salt bridge. AK has also been identified in yeast and bacterial species. Two enzymes are also known to be related to the AK family. These are yeast uridine monophosphokinase and slime mold UMP-CMP kinase. Despite having similar structural features and functional mechanisms, different intracellular localization and tissue distributions of AK isoforms explains their specific properties and pathological implications.

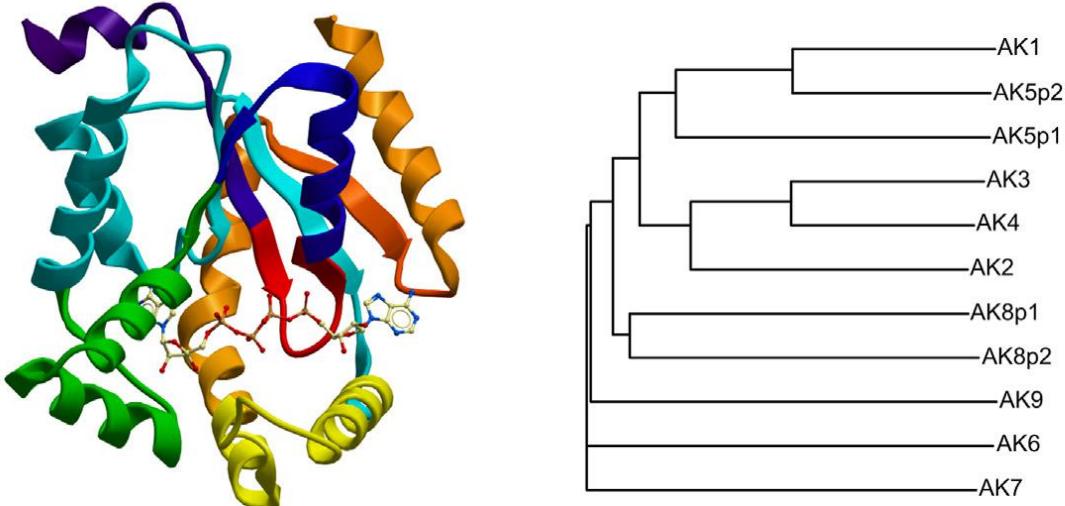
### V.iii A deeper view of Human AK isoforms

#### V.iii.1 Adenylate Kinase 1 (AK1)

AK1 (Accession Number: AAH01116) is quite abundant as it can be found in almost every tissue of human body, It is made of 194 residues, and is located in



(a) Amino acid sequence of human AK isoforms



(b) Crystal structure of human AK1 complexed with AP5A inhibitor

(c) Phylogenetic tree of human AK isoforms

Figure 10: Sequence, crystal structure and genetic tree of human AK isoforms. Red, yellow and green colored areas represents CORE, LID and NMP domains of AK respectively [1]

cytosol within the cells, with its gene present on chromosome 9 at location 9q34.11 (NCBI Gene ID: 203) [1, 4]. Highest concentration of AK1 is found in the brain, skeletal muscles and red blood cells (erythrocytes) [20]. AK1 can use all nucleoside triphosphates as phosphate donors, and hence shows no donor specificity. Its main substrate is AMP, though it can also phosphorylate dAMP to some extent [19, 21]. Human AK1 is quite similar to that of other organisms, and exhibits 88% and 60% similarity with *Mus musculus* and *Caenorhabditis elegans* respectively.

Deficiency of AK in red blood cells leads to a rare condition associated with *haemolytic anaemia*. Low AK1 enzymatic activity forms the basis for diagnosis of such conditions, which are found to be rarely linked with genetic defects.

## Case Studies

- A single base mutation of arginine 128 to tryptophan was identified in a Japanese patient with haemolytic anaemia, which resulted in a mutated AK1 enzyme with 44% activity, change in electrophoretic mobility and reduced thermal stability. However, mother and brother of the same patient lacked any symptom of anemia despite having same the AK1 mutation in erythrocytes. It suggest that red blood cells can withstand extreme AK1 deficiency without major consequences in some cases [22].
- Single substitution of tyrosine-164 residue of AK1 by cysteine in case of an Italian child resulted in a mutated and fundamentally dysfunctional enzyme which lead to haemolytic anaemia. Resultant change in AK1 activity can attributed to new disulphide linkages possible due to the presence of extra cysteine residue [22, 23].
- A homozygous mutation of AK1 that resulted in truncated enzyme with absolutely no catalytic activity was reported in case of two siblings with chronic haemolytic anaemia. Such siblings with AK1 deficiency showed psychomotor dysfunction, language delay and poor learning capacity [24].

As shown by many studies, total loss of AK1 enzymatic activity, in some cases can be made up by the presence of other AK1 isoforms and metabolic pathways. Under most of normal circumstances, deficiency of AK1 in heart could

be compensated by increasing metabolic flow through the remaining glycolysis pathways and AK isoforms, which allows for an adaptive achievement of energy homeostasis [25].

### V.iii.2 Adenylate Kinase 2 (AK2)

AK2 (Accession Number: AAC52061) is present inside the intermembranal space of mitochondria within the cell, with its gene present on chromosome 1 at location 1p35.1 (NCBI Gene ID: 204) [4, 26]. It consists of 239 residues and is found abundantly in heart, liver, kidney, and to a lesser extent in skeletal muscles and lungs. Messenger RNA (mRNA) of AK2 is abundant in heart, skeletal muscle, pancreas and liver, with moderate concentration in brain, kidney and placenta, and even lower concentrations in lung tissues [20, 27]. Like AK1, it shows no specificity towards any particular phosphate donor, and can use any nucleoside triphosphate (NTPs). It is however specific to AMP substrate [21]. Human AK2 displays high similarity with other isoforms, and is identical to an extent of 45% with human AK3, 40% with human AK4, 39% and 54% with AK from *Drosophila melanogaster* and *Escherichia coli* respectively [28].

Deficiency of AK2 can cause several diseases of varying order. However, more life threatening is the mutation of AK2 gene which can lead to *reticular dysgenesis*, a specific type of Severe Combined Immunodeficiency (SCID) in inborn humans. It is characterized by the absence of certain type of white blood cells (lymphocytes, granulocytes etc), and the lack of natural and adaptive cellular immunity, causing fatal septicemia within few days of birth. AK2 deficiency is also associated with sensorineural deafness (hearing loss caused by damage to auditory nerve) [29].

AK2's presence in the mitochondria suggests that it plays a crucial part in supplying the energy needed for the development of hematopoietic cells (blood stem cells that can develop into any type of blood cells including rbc, wbc and platelets) and in regulating cell death (or apoptosis) [30]. AK2 forms complex with Caspase-10 and FADD (Fas-Associated protein with Death Domain), which activates a loop responsible for cell apoptosis [31].

### V.iii.3 Adenylate Kinase 3 (AK3)

AK3 (Accession Number: AAH13771) is composed of 227 residues and is present in the mitochondrial matrix, with highest concentration levels in skeletal muscle, brain, kidney, liver, and heart of the human body [32]. Its gene is present on chromosome 9 at 9p24.1 site (NCBI Gene ID: 50808). Unlike AK1 and AK2, AK3 can only use Guanosine triphosphate (GTP) and Inosine triphosphate (ITP) as phosphate donor to phosphorylate AMP. Hence, it shows both donor and substrate specificity [21]. AK3 can be crystallized as a standalone monomeric protein without any co-factor.

### V.iii.4 Adenylate Kinase 4 (AK4)

AK4 (Accession Number: P27144) is composed of 223 residues (4 less than AK3) and is present in mitochondrial matrix (like AK3). Its gene is present on chromosome 1 at 1p31.3 (NCBI Gene ID: 205). It was originally discovered in 1992 and was initially named AK3 (owing to its 99% sequence identity with bovine AK3) but was later renamed to AK4 after the discovery of mouse and rat AK4 [33]. AK4 is mainly found in kidney, brain, liver, heart and other organs rich in mitochondria. It can only use ATP and GTP as phosphate donors, and has the ability to phosphorylate AMP, CMP (Cytidine monophosphate), dAMP and dCMP substrates. It was shown that the first 11 residues from N-terminal of AK4 plays an important role in transporting the enzyme to mitochondrial matrix. These residues forms the "*mitochondrial import sequence*" of AK4, which is very close to phosphate binding P-loop region. Studies indicate that this sequence is *not* cleaved as AK4 enters the mitochondrial matrix, which explains how AK4 retains its enzymatic activity after the transport [34].

Studies suggest that AK4 levels increase in response to oxidative stress like *Hypoxia* (a state in which tissues does not have enough oxygen to maintain normal homeostasis) and *Amyotrophic Lateral Sclerosis* (ALS, a neurodegenerative disorder with associated oxidative stress) [35].

### V.iii.5 Adenylate Kinase 5 (AK5)

As opposed to other AK isoenzymes, AK5 (Accession Number: AAH33896) is only found in the brain, with its gene present in chromosome 1 at site 1p31.1 (NCBI Gene ID: 26289). It consists of 537 residues and found in two main variants, where one of the variant has an extra 26 residues long chain attached to N-terminal that acts as "nuclear import sequence". This additional sequence allows AK5 to diffuse from cytosol to nucleus without loosing activity. Thus, AK5 can be found in either cytosol or both nucleus and cytosol of the cell. AK5 is divided into two independent functional subdomains, namely AK5p1 and AK5p2. Even when AK5p1 or AK5p2 are found separately, they can still catalyze phosphorylation reaction similar to full length AK5 enzyme [36]. Exactly like AK4, AK5 can only use ATP and GTP as phosphate donors for the phosphorylation of AMP, CMP, dAMP and dCMP substrates [37].

### V.iii.6 Adenylate Kinase 6 (AK6)

AK6 (Accession Number: AAO16520) is 536 residues long enzyme, and is extremely similar to AK5 (99% identical). It is the only human AK isoforms that is purely nuclear with regard to its intracellular localization. However, tissue distribution of AK6 is not fully known, although every normal tissue has moderate to high concentration of AK6. Its gene is present on chromosome 5 at long arm 5q13.2 (NCBI Gene ID: 102157402).

AK6 can use all NTPs and dNTPs as phosphate donors (Cytidine and Uridine triphosphate are preferred) to phosphorylate AMP, CMP, dAMP and dCMP substrates. AK6's nuclear subcellular location and broad activity makes it highly versatile, and suggests its central role in phosphotransfer reactions and energy homeostasis. One of its striking features is that it can regulate ATP/ADP and GTP/GDP ratios simultaneously [38]. Recent studies have suggested that AK6 shows an extended catalytic activity in regulating the assembly and disassembly of Cajal bodies (CBs, sub-nuclear structures present in eukaryotic cells) of human cell nuclei [39].

### V.iii.7 Adenylate Kinase 7 (AK7)

AK7 (Accession Number: NP\_689540) is quite different from other human AK isoforms. It is located in cytosol within the cell, with its gene present on the long arm of chromosome 14 at 14q32.2 (NCBI Gene ID: 122481), contradictory to other cytosolic AKs (AK1, AK5, AK8) where gene is mostly found either on chromosome 1 or 9. The cDNA (complementary DNA) of AK7 was shown to produce a 656 residues long protein, while NCBI database shows that AK7 gene should produce a protein with 723 residues, which is significantly longer than other AK isoforms. The long length of AK7 is attributed to "stop codon" (a trinucleotide sequence in DNA or mRNA that signals the end of protein synthesis) present at the end of its gene, which suggests the existence of more than one AK7 transcript variants. AK7 can phosphorylate AMP, CMP, dAMP and dCMP using ATP as phosphate donor, but cannot phosphorylate dAMP when GTP is used in place of ATP as phosphate donor [19].

AK7 is highly conserved across eukaryotes, It contains three structural domains that are conserved across species. First is the *WcaG* domain that spans from residue 147 to 310 (near N-terminal of protein), and is a member of nucleoside diphosphate sugar epimerase family. Second is the core *adenylate kinase* domain which spans from residue 367 to 548, and the third is *Dpy30* motif (a short sequence within a protein or DNA that performs a specific function) that spans from residue 678 to 720 (near C-terminal) which might play a role in dimerisation.

AK7 is almost exclusively found in tissues with epithelium rich in cilia such as trachea, skeletal muscle, testis, mammary gland and lungs. Thus, AK7 enzymatic activity and psychological impact is related to its specific tissue distribution and cilia function [40]. Motile cilia requires huge amount of energy supplied by the hydrolysis of ATP. Anomalies in energy supply or ciliary function can cause *primary ciliary dyskinesia* (family of recurrent heterogeneous diseases that cause respiratory infections). One such anomaly is cytosolic AK7 deficiency, which disturbs cellular energy homeostasis. In a model with AK7 deficient mouse, pathological symptoms characteristic to ciliary dyskinesia such as low ciliary beat frequency, microtubular and spermatogenesis (production of sperm) disorders and accumulation of mucus were observed [40]. In humans,

primary ciliary dyskinesia can lead to failure of lungs in adults and birth defects such as retinal degeneration, mental retardation, hydrocephalus (a neurological disorder) and hearing loss. Furthermore, sperm dysmotility in males and reduced ciliary movement in fallopian tubes of females can cause fertility disorders [41, 42].

#### **V.iii.8 Adenylate Kinase 8 (AK8)**

AK8 (Accession Number: NP\_689785) is a 479 residues long protein present in cytosol within the cell. Its gene is found on the long arm of chromosome 9 at site 9q34.13 (NCBI Gene ID: 158067). Much like AK7, AK8 is also well conserved across eukaryotes and exhibits identical phosphate donor and substrate specificity: uses ATP to phosphorylate AMP, CMP, dAMP and dCMP and GTP to phosphorylate AMP, CMP and dCMP [19]. Like AK5, AK8 also has two independent functional subdomains, namely AK8p1 and AK8p2. Both the domains have identical phosphorylation ability, which allows for the full length AK8 to have two functionally active domains within a single protein chain. This explains high phosphorylation efficiency of AK8.

AK8 is mostly found in pancreas, trachea, testis and liver of the human body, with its gene playing a crucial role in the regulation of epithelial cell migration [43].

#### **V.iii.9 Adenylate Kinase 9 (AK9)**

AK9 is the more recent addition to the human AK isoform family. Its gene is found on chromosome 6 at 6q21 (NCBI Gene ID: 221264). It is majorly found with high levels in trachea, testis, thymus, mammary gland and pituitary gland, with moderate levels in uterus, pharynx, lymph nodes, spleen and brain in the human body. However, intracellular location of AK9 is still not clear. GFP fusion studies showed that AK9 lacks localized target signals within the cell, and instead exhibits a diffused fluorescence pattern which indicates that *AK9 can freely diffuse from cytosol to nucleus* [1, 18].

Human AK9 exists in two isomeric forms, namely AK9i1 and AK9i2. AK9i1 (Accession Number: NP\_001138600) is a 1911 residues long protein, and shows similarity with AK9 from other species. Database searches indicate 30% similarity between AK9i1 and AK5 enzyme from *Glycine max*. AK9i2 (Accession Number:

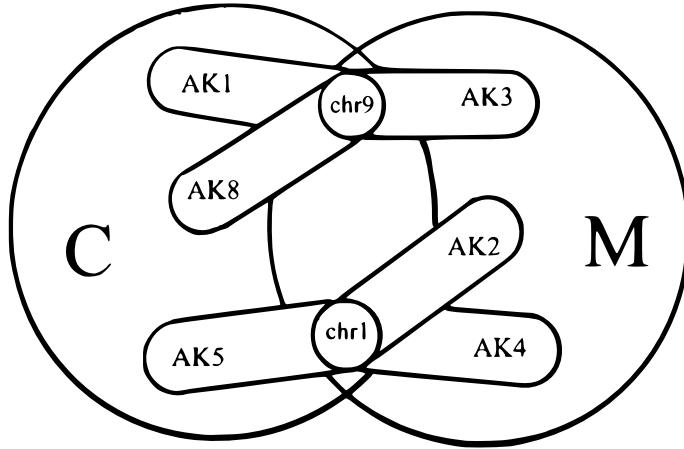


Figure 11: Cytosolic and mitochondrial AKs with chromosomes containing their respective genes (only exception is the gene of cytosolic AK7, present on chromosome 14) [4]. Abbreviations  $\Rightarrow$  C: Cytosol, M: Mitochondria, chr: Chromosome

NP\_659462) consists of significantly lower 421 residues, and shows 26% and 29% similarity with AK1 from *Pseudomonas aeruginosa* and *Plasmodium falciparum* respectively [4].

AK9 can use either of ATP or GTP as phosphate donors to phosphorylate AMP, CMP, dAMP and dCMP. However, when GTP is used as the donor, AK9 shows high specificity for CMP substrate. In addition to nucleoside monophosphate kinase, AK9 can sometimes also catalyze phosphorylation of nucleoside diphosphates i,e AK9 can also act as nucleoside diphosphate kinase [18]. This is explained further in section VI.ii.

## VI Functions of Adenylate Kinase

### VI.i AK Shuttle

Within the mitochondrial and myofibrillar compartments of the cell, Adenylate Kinase is present in two major isoforms: Cytosolic Adenylate Kinase (or AK1)

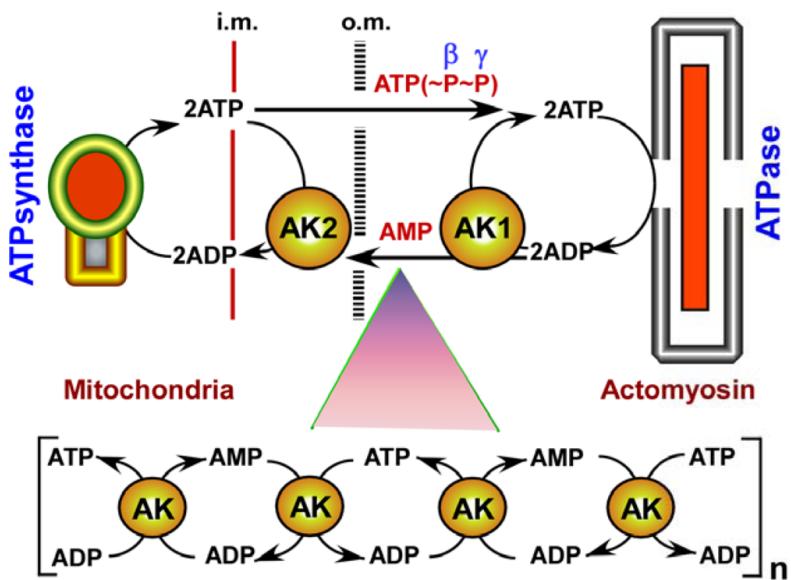


Figure 12: Transfer of high energy  $\beta$  and  $\gamma$  phosphoryls of ATP from production to consumption site via ATP shuttle. (i.m: inner membrane, o.m outer membrane) [44]

and mitochondrial Adenylate Kinase (or AK2), and it allows for the transfer and use of energy of two high-energy phosphoryls ( $\beta$  and  $\gamma$ ) of a single ATP molecule. In effect, cytosolic Adenylate Kinase (or AK1) *shuttles* ATP to sites of high energy consumption and converts ADP molecules generated as by product of these androgenic reactions to ATP and AMP. The AMP then signals mitochondrial Adenylate Kinase (AK2) to generate two molecules of ADP at the inner membrane (i.m) of mitochondria [44]. This is shown in upper portion of figure 12.

The transfer of ATP and AMP from ATP-production to ATP-consumption site (and reverse) within intracellular environment may involve multiple phosphotransfer stages in a sequential manner (like a sequential relay). This results in propagation of ATP and AMP flux through a series of adenylate kinase molecules, and is illustrated in lower part of figure 12. Thus, the series of AK molecules that facilitates conduction of metabolic flux (or ATP-AMP flux) can be thought as “*bucket-brigade*” [44]. This type of intracellular conduction along a

series of AK molecules may change local metabolic flux without any apparent change in global metabolic (or ATP-AMP) concentration of the cell. This whole process is crucial for overall energy homeostasis of the cell.

## VI.ii Nucleoside Diphosphate Kinases (NDPKs)

### VI.ii.1 Introduction and Mechanism

NDPKs are enzymes that catalyze the reversible phosphoryl transfer from a donor to nucleoside diphosphate (NDP) substrate, producing corresponding nucleoside triphosphate (NTP). Family of genes that encode NDPKs are called *nm23*. So far, ten such genes have been identified in humans and are designated *nm23-H1* to *nm23-H9* with the last gene known as *RP2* [45]. The two major features that differentiates NDPKs from nucleoside kinases (NPs) and nucleoside monophosphate kinases (NMPKs) involved in NTP (or dNTP) synthesis are

- NDPKs do *not* have a p-loop motif in phosphate donor binding site.
- NDPKs are *not* specific to any particular substrate. They can accept pyrimidine and purine ribo and deoxyribo nucleoside diphosphates as substrates.

NDPKs follow a ping-pong mechanism illustrated in figure 13. The steps are as follows [46]

1. NDPK binds with the phosphate donor  $\text{NTP}_{(\text{d})}$
2.  $\gamma$  phosphate of  $\text{NTP}_{(\text{d})}$  is transferred to histidine residue of the NDPK, resulting in a high energy phosphoenzyme intermediate NDPK-His-P bound to  $\text{NDP}_{(\text{d})}$ .
3.  $\text{NDP}_{(\text{d})}$  detaches from the enzyme which triggers conformational change that facilitates the binding of substrate  $\text{NDP}_{(\text{s})}$  or  $\text{dNDP}_{(\text{s})}$  to the phosphoenzyme intermediate.
4. Transfer of phosphoryl group from NDPK-His-P to the bound  $\text{NDP}_{(\text{s})}$  or  $\text{dNDP}_{(\text{s})}$  substrate results in  $\text{NTP}_{(\text{s})}$  or  $\text{dNTP}_{(\text{s})}$ .
5. Product  $\text{NTP}_{(\text{s})}$  or  $\text{dNTP}_{(\text{s})}$  is released, resulting in enzyme back to its initial open conformation ready to catalyze a new cycle.

NDPKs are shown to play an important role in many biological processes such as protein synthesis, metabolic signaling, cellular development, proliferation, differentiation and apoptosis [47].

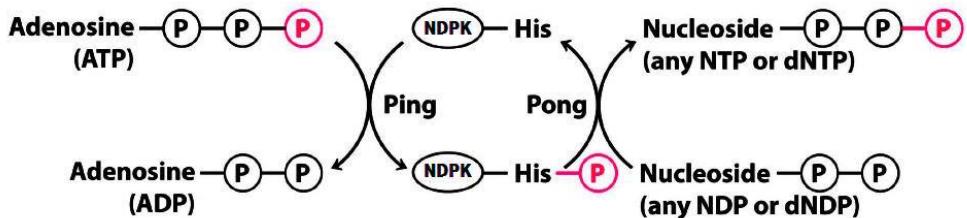


Figure 13: Ping-Pong mechanism followed by NDPKs for the phosphorylation of NDP or dNDP substrate. This example use ATP as phosphate donor [46]

### VI.ii.2 Adenylate Kinase as NDPKs

Recent studies showed that in addition to defining NMPK activity, AK9 also has substantial NDPK activity. AK9 can catalyze the formation of nucleoside triphosphates such as ATP, dATP, CTP, dCTP, UTP, GTP, dGTP and Thymidine triphosphate (TTP) using corresponding nucleoside diphosphates irrespective of the phosphate donor. However, its NDP substrate specificity slightly varies in the order CDP > UDP > ADP > GDP > TDP [1]. Likewise, other human AK isoenzymes can also convert majority of NDPs and dNDPs to corresponding NTPs and dNTPs. In some cases, enzyme shows restricted NDP (or dNDP) substrate binding ability. One example is of AK8 which cannot phosphorylate UDP when ATP is used as phosphate donor. However, such cases can easily be bypassed by using some alternate phosphate donor such as GTP. AK8 can phosphorylate UDP when GTP is used as phosphate donor in place of ATP.

As evident from Table 4, human AK isoforms are quite specific to NMP and dNMP substrates in case of NMPK activity. However, this substrate specificity is significantly changed and reduced when NDP and dNDP substrates are used in case of NDPK activity. This is because active site of AK isoenzymes is optimized to bind with specific NMP or dNMP substrates. NDP (and dNDP) substrates

usually do not fit very well in those active sites and can easily attack  $\gamma$  phosphoryl group of the donor molecule, resulting in poor NDP specificity [18]. The NDPK activity of AK family was studied in a bacteria, *Ureaplasma parvum* that lacks NDPK gene. When AKs from *Ureaplasma parvum* were cloned and inserted into a mutated *Escherichia coli* with disrupted NDPK activity, newly inserted AK showed NDPK activity. Deficiency of NDPKs was compensated by the presence of NMPKs such as AK [48, 49].

## VI.iii Intracellular Synthesis of Purine Nucleotides

### VI.iii.1 Importance of Nucleotides

Many different nucleotides (and their derivatives) are present in all types of cells, where they play important role in numerous metabolic processes necessary for healthy cellular growth and function.

1. **Nucleic Acids:** Nucleic acids such as RNA and DNA are polymers of ribonucleotide and deoxyribonucleotide monomeric units respectively. Furthermore, adenine nucleotide forms the basis of coenzymes such as Flavin Adenine Dinucleotide (FAD), Coenzyme-A (CoA) and Nicotinamide Adenine Dinucleotide (NAD), which play important role in electron, hydride and acyl group transfer reactions in metabolism [50].
2. **Energy Carriers:** The primary chemical energy source (or *energy currency*) for the cell is ATP, which also acts as a phosphate donor and as a driving force for numerous metabolic processes within the cells.
3. **Metabolic Signaling:** AMP and cyclic AMP (cAMP) are ubiquitous intracellular signaling molecules, whose level changes in response to various chemical, physiological and hormonal stimuli. cAMP regulates many cAMP-controlled protein kinases, and thereby controls the activation and inhibition of many enzymes, genetic expressions, calcium channels with cAMP receptors as well as metabolic processes such as glycogenesis and glycogenolysis [51]. Likewise, change in AMP levels affects many AMP-controlled components such as metabolic sensors, glycolytic and glycogenolytic metabolic processes, kinases protein, channels for active

potassium and calcium transport etc. All together, cAMP and AMP regulates metabolic pathways and the balance between cellular energy production and consumption [44, 51].

**4. Pharmacological use:** Blood coagulation via platelet aggregation is greatly affected by intracellular ADP levels. Hence, drugs targeting ADP are supposed to treat blood clotting disorders such as Hemophilia [52]. *Nucleoside analogues* are major class of novel drugs that resembles natural nucleosides and depend on intracellular phosphorylation for their activity. They are useful in treatment of viral infections, cancer and other malignant diseases. They are described with detail in section VI.iv.

Since the cell membrane does not contain any nucleotide carrier protein and negatively charged phosphate groups resists diffusion over the membrane, all type of nucleotides must be produced inside the cell. Defects in intracellular nucleotide synthetic pathways can lead to several diseases. Deep understanding of such processes is therefor important for both the diagnosis and treatment.

Mammalian cells synthesize purine nucleotides mainly via two pathways: *de-novo* pathway (chemical synthesis of complex molecules from simple ones) and *salvage* pathway (chemical synthesis that uses left over intermediates and by-products from other destructive processes of similar molecule) [1].

### VI.iii.2 The *de-novo* pathway

The *de-novo* pathway of purine nucleotide synthesis is the major source of DNA transcription and replication in developing cells. It uses Phosphoribosyl pyrophosphate (PRPP) as the source of ribose 5'-phosphate, which together with amino acids, CO<sub>2</sub> and N10-formyltetrahydrofolate (simple molecules as starting point) leads to the formation of inosine monophosphate (IMP). IMP then serves as precursor for important nucleoside monophosphates such as AMP and GMP (guanosine monophosphate). AMP and GMP are then phosphorylated by Adenylate Kinase (AK) and Guanylate Kinase (GUK) to ADP and GDP respectively. ADP and GDP can be used to produce ATP and GTP using nucleoside diphosphate kinases (NDPKs) respectively. Alternatively, they can also be reduced to dADP and dGDP (deoxyribonucleoside diphosphates) by the

action of ribonucleoside reductase. Like ADP and GDP, dADP and dGDP can also be phosphorylated by NDPKs to give dATP and dGTP [53, 54].

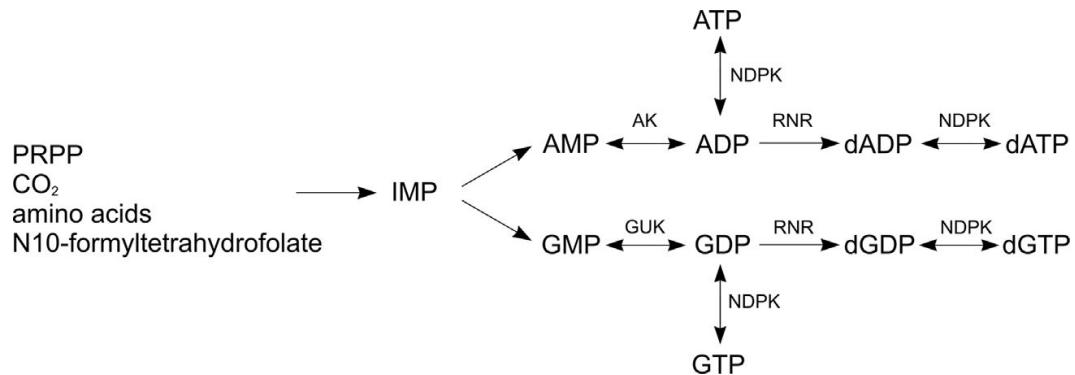


Figure 14: The *de-novo* synthesis of purine nucleotides inside a mammalian cell<sup>[1]</sup>.

### VI.iii.3 The *Salvage* Pathway

The *salvage* pathway is essential for DNA repair, replication and transcription in damaged cells. It uses nucleoside precursors that are either derived from free bases or left as by products of RNA and DNA degradation and following dephosphorylation of their monomeric units (ribo and deoxyribo nucleoside triphosphates respectively). In essence, it *salvages* left overs molecules for the production of nucleotides. In case nucleoside precursors are outside the cellular membrane, they are either actively transported or passively diffused across the cell membrane via nucleoside carrier proteins. Once inside the cell, ribo and deoxyribo nucleoside kinases phosphorylate ribo and deoxyribo nucleosides to corresponding monophosphates (NMP and dNMP) respectively. As in case of *de-novo* pathway, nucleoside monophosphates are converted to diphosphates by the action of NMPKs. They can then either be directly phosphorylated by NDPKs to give ribo and deoxyribo nucleoside triphosphates or can be acted upon by ribonucleoside reductases followed by NDPKs to give deoxyribonucleoside triphosphates [55].

## VI.iv Nucleoside Analogues

### VI.iv.1 Introduction, Mechanism and Effect

Nucleoside Analogues are a class of compounds that resembles the structure of natural nucleosides, which are the building blocks of nucleic acids like DNA and RNA. They are used in the treatment of several malignant diseases (various form of cancers such as leukaemia, lung cancer, solid tumors), viral infections (influenza, hepatitis B and C, HIV, herpes etc) and other autoimmune diseases. Overall process, mechanism of action and effects of nucleoside analogues is described below.

- **Administration:** Nucleoside analogues are usually administered as monophosphates to increase their solubility. Before entering the cell, they are dephosphorylated back to nucleoside form by the action of various dephosphorylating enzymes such as nucleotidases.
- **Transport:** Since nucleoside analogues are usually hydrophilic molecules, they require special *nucleoside transport proteins* to cross the cellular membrane and enter the cell. Hence, specificity of a nucleoside analogue towards particular type of cells and sensitivity of a cell towards a particular nucleoside analogue depends on the intramembranal concentration and tissue distribution of nucleoside transport proteins [56].
- **Intracellular Activation:** Once inside the cell, nucleoside analogues are *activated* by phosphorylation to nucleoside triphosphate form in a stepwise manner.
  1. Nucleoside analogue is phosphorylated to monophosphate form by the action of ribo and deoxyribo nucleoside kinases such as cytidine and guanosine kinase (CK, GK, dCK and dGK: d represents deoxyribo). However, dephosphorylating action of various nucleotidases present in the cell counteracts this process. Thus, this step is the *rate-limiting step* for nucleoside analogue activation process [57, 58].
  2. Nucleoside analogue monophosphates resulting from the two opposing processing in the first step is then *efficiently* phosphorylated by the action of several AK isoforms to their corresponding diphosphates. This

is shown to be true in case of anticancer drugs deoxycytidine (dCyd) and deoxyadenosine (dAdo) using thin layer chromatography [1, 56].

3. Finally, nucleoside analogue diphosphates are phosphorylated to triphosphates by the action of NDPKs. In case NDPKs are absent or of low activity, this job can be taken by NMPKs (as described in section VI.ii).
- **Incorporation:** After activation to triphosphate form, nucleoside analogues are then incorporated into DNA and RNA of the viral or malignant cell by the action of nucleic acid synthesis, replication or repair mechanisms. If a nucleoside analogue contains both 3' and 5' hydroxyl groups, it can link with the DNA backbone from one of the hydroxyl site, and the remaining hydroxyl allows DNA polymerase to extend the DNA chain upon newly inserted analogue. On the contrary, if the nucleoside analogue lacks 3' hydroxyl group, it can only link with the DNA in a terminal fashion i.e it terminates the DNA chain. In either case, incorporation of nucleoside analogue leads to mutation of the DNA chain causing errors in DNA replication and transcription processes.
  - **Effect:** Incorporation of nucleoside analogue into the nuclear or mitochondrial DNA (mtDNA) causes defective DNA replication and protein synthesis leading to the loss of cellular components (nucleus, mitochondria) integrity. If the mtDNA mutation is irreparable, mitochondria releases cytochrome c protein into the cytosol that initiates cell apoptosis cascade leading to cell death. Thus, *nucleoside analogues are cytotoxins*. Furthermore, disruption of mtDNA transcription causes reduced and faulty synthesis of mitochondrial proteins which are crucial for electron transport and other energy demanding processes such as oxidative phosphorylation. Some of the nucleoside analogues also inhibit enzymes that are required for the intracellular synthesis of pyrimidine and purine nucleotides (described in section VI.iii.2 and VI.iii.3). This reduces the availability of dNTPs required for the synthesis and replication of DNA [25, 59].
  - **Side Effects:** Since nucleoside analogues are cytotoxins that disrupts

mtDNA, their long term usage can cause disorders that resemble genetic mutations in the mitochondria. Example of such multi-organ disorders are lactic acidosis, neuropathy, cardiomyopathy, and organ (liver, bone marrow, pancreas etc) failure [60].

#### VI.iv.2 Examples

**Cladribine:** It is an analogue of deoxy-Adenosine (dAdo) with the formula 2-Chloro, 2-deoxy-Adenosine (**CdA**) (figure 15a). Unlike dAdo, it resists deamination action of adenosine deaminase present inside the cell. It is phosphorylated by the action of deoxy-Cytidine Kinase (dCK) and deoxy-Guanosine Kinase (dGK). Hence, cellular sensitivity to CdA depends on the intracellular ratio of dCK and nucleotidases competing in the first phosphorylation step. Lymphocytes (type of white blood cells) have high concentration of dCK compared to nucleotidases, and are highly sensitive to CdA [61]. High accumulation of CdA triphosphate (CdATP) in infected lymphocytes leads to the death of cancerous immune cells. For this reason, CdA is used in the treatment of autoimmune diseases such as chronic lymphocytic leukaemia, multiple sclerosis (MS) and hairy cell leukaemia [62, 63].

**Fludarabine:** It is another structural analogue of dAdo with formula 2-Fluoro, 9- $\beta$ -d-arabinofuranosyl-Adenine (**FaraA**) (figure 15b). It is also a cytotoxin like CdA and is used against autoimmune disorders [64]. DNA repair mechanisms incorporate FaraATP (or CdATP) into DNA sequence, which causes multiple breaks in the single strand of DNA. This irreparable mutation of DNA eventually leads to cell apoptosis.

**Gemcitabine:** It is an analogue of deoxy-Cytidine (dCyd) with the formula 2',2'-Difluorodeoxycytidine (**dFdC**) (figure 15c). It is an approved drug for the treatment of viral infections and solid tumors in neck, bladder, breast, pancreas, ovary and lung cancers. dFdC has high substrate affinity for nucleoside transport proteins and deoxy-Cytidine Kinase (dCK). Consequently, dFdC is easily transported across the cell membrane and phosphorylated to triphosphate dFdCTP, which can be incorporated into DNA as well as RNA. These traits

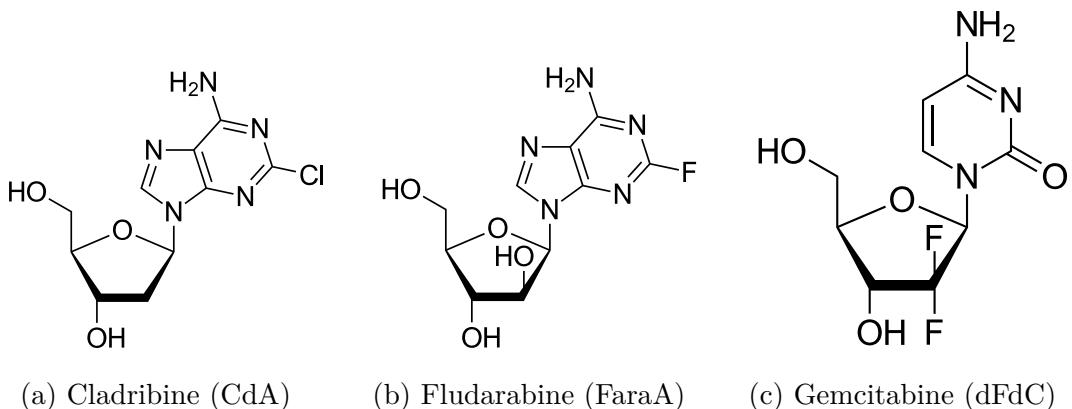


Figure 15: Nucleoside analogues

increase the cytotoxicity of dFdCTP by facilitating its effective accumulation and prolonged retention in cancer cells [56, 65].

## VII Conclusion

Adenylate Kinase is a widespread family of isoenzymes, present in almost all living organisms due to its critical role in cellular energy metabolism and homeostasis. It is the only intracellular enzyme that can catalyze the interconversion of various adenine nucleotides, essential for nucleic acid synthesis and repair, cellular energy production and transfer among other processes. The structure, mechanism of action, enzymatic kinetics and physiological role of AK have been extensively studied. Structurally, AK is composed of 3 domains: CORE, LID and NMP, where CORE domain contains the active site and is highly conserved across species while LID and NMP are variable surface loops and provide binding sites for phosphate donor and substrate acceptor. During the phosphate transfer, AK adopts wide range of conformations, with *open* and *closed* being the most favorable. LID and NMP domains are flexible and undergo significant conformation changes upon binding and release of donor and substrate molecules. On the contrary, CORE domain is conformationally stable and facilitates phosphoryl transfer upon binding. MD simulations are proved to be useful in tracking the structure and thermodynamics of various conformations

acquired by AK along the way.

Various assays have been developed to measure the activity of AK under the influence of activators/inhibitors and other factors, most of which conventionally uses coupled enzymatic reactions. Spectrophotometric assays employing pH based indicators provide one-step method for sensitive, continuous and real time monitoring of AK activity.

Nine human AK isoforms have been identified so far, which differ in substrate and phosphate donor specificity, kinetics, subcellular localization and tissue distribution leading to different physiological functions and therapeutic impact. For instance, AK1 is present in cytosol of muscle and brain cells, and plays crucial role in energy metabolism during muscle contraction and neurotransmitter release. AK2 is present in mitochondrial intermembrane and regulates adenine nucleotide levels in mitochondria, while AK3 is present in mitochondrial matrix where it generates ATP via oxidative phosphorylation. Contrary to this, many essential enzymes only exist in one form, which makes the existence of many isoforms of AK indicative of its important role in diverse array of cellular processes such as Nucleoside mono and di-phosphate kinases (NMPKs and NDPKs), AK shuttle, intracellular synthesis of purine nucleotides, metabolic energy signaling and sensing. The need of so many isoforms of the same enzyme in humans is a question that still requires further research.

Dysregulation of AK activity is shown to be associated with medical conditions such as heart failure, neurodegenerative disorders and malignant diseases. For example, deficiency of AK1 is implicated in *haemolytic anaemia*, dysfunction of AK2 can cause *reticular dysgenesis*, AK7 deficiency is associated with *primary ciliary dyskinesia*. Therefore, therapeutic interventions targeting AK activity have potential to treat such diseases. One example is of Nucleoside analogues, which are drugs that resemble natural nucleosides and depends on intracellular phosphorylation by AK for their activation. They are effective in the treatment of viral infections, malignant diseases and auto-immune disorders. Since ATP is the primary "*energy currency*" of the cell, increasing its production for various cellular functions is of high interest. Regulation of ATP generating process in case of various diseases is an important and challenging subject of research. Further studies in AK are therefore crucial to better understand its

complex phosphoryl transfer mechanism and role in cellular processes, which can provide insights for the development of novel therapeutic strategies.

## References

- (1) Panayiotou, C.; Solaroli, N.; Karlsson, A. The many isoforms of human adenylate kinases. *The international journal of biochemistry and cell biology* **2014**, *49*, 75–83.
- (2) Krishnamurthy, H.; Lou, H.; Kimple, A.; Vieille, C.; Cukier, R. I. Associative mechanism for phosphoryl transfer: A molecular dynamics simulation of *Escherichia coli* adenylate kinase complexed with its substrates. *Proteins: Structure, Function, and Bioinformatics* **2005**, *58*, DOI: [10.1002/prot.20301](https://doi.org/10.1002/prot.20301).
- (3) Chang, H.-Y.; Fu, C.-Y. Adenylate Kinase. *Encyclopedia of Food Microbiology: Second Edition* **2014**, 18–23, DOI: [10.1016/B978-0-12-384730-0.00003-3](https://doi.org/10.1016/B978-0-12-384730-0.00003-3).
- (4) Ionescu, M. I. Adenylate kinase: a ubiquitous enzyme correlated with medical conditions. *The Protein Journal* **2019**, *38*, 120–133.
- (5) Formoso, E.; Limongelli, V.; Parrinello, M. Energetics and structural characterization of the large-scale functional motion of adenylate kinase. *Scientific reports* **2015**, *5*, 8425.
- (6) Rundqvist, L.; den, J.; Sparrman, T.; Wallgren, M.; Olsson, U.; Wolf-Watz, M. Noncooperative folding of subdomains in adenylate kinase. *Biochemistry* **2009**, *48*, 1911–1927.
- (7) Olsson, U.; Wolf-Watz, M. Overlap between folding and functional energy landscapes for adenylate kinase conformational change. *Nature communications* **2010**, *1*, 111.
- (8) Reinstein, J.; Gilles, A.-M.; Rose, T.; Wittinghofer, A.; Saint Girons, I.; Bârzu, O.; Surewicz, W. K.; Mantsch, H. H. Structural and catalytic role of arginine 88 in *Escherichia coli* adenylate kinase as evidenced by chemical modification and site-directed mutagenesis. *Journal of Biological Chemistry* **1989**, *264*, 8107–8112.
- (9) Bücher, T.; Pfleiderer, G. In *Methods in Enzymology*, Vol. 1; Academic Press: 1955, pp 435–440, DOI: [10.1016/0076-6879\(55\)01071-9](https://doi.org/10.1016/0076-6879(55)01071-9).
- (10) Oliver, I. T. A spectrophotometric method for the determination of creative phosphokinase and myokinase. *Biochemical Journal* **1955**, *61*, 116–122, DOI: [10.1042/bj0610116](https://doi.org/10.1042/bj0610116).

- (11) Chiu, C.-S.; Su, S.; Russell, P. J. Adenylate kinase from baker's yeast. I. Purification and intracellular location. *Biochimica et Biophysica Acta (BBA) - Enzymology* **1967**, *132*, 361–369, DOI: [10.1016/0005-2744\(67\)90155-6](https://doi.org/10.1016/0005-2744(67)90155-6).
- (12) Song, K.; Wang, Y.; Li, Y.; Ding, C.; Cai, R.; Tao, G.; Zhao, P.; Xia, Q.; He, H. A Convenient, Rapid, Sensitive, and Reliable Spectrophotometric Assay for Adenylate Kinase Activity. *Molecules* **2019**, *24*, DOI: [10.3390/molecules24040663](https://doi.org/10.3390/molecules24040663).
- (13) Yu, Z.; Pan, J.; Zhou, H. A direct continuous pH-spectrophotometric assay for arginine kinase activity. *Protein and peptide letters* **2002**, *9*, 545–552, DOI: [10.2174/0929866023408382](https://doi.org/10.2174/0929866023408382).
- (14) Dhale, M. A.; Mohan-Kumari, H. P. A comparative rapid and sensitive method to screen l-asparaginase producing fungi. *Journal of Microbiological Methods* **2014**, *102*, 66–68, DOI: [10.1016/j.mimet.2014.04.010](https://doi.org/10.1016/j.mimet.2014.04.010).
- (15) Colowick, S. P. In Methods in Enzymology, Vol. 2; Academic Press: 1955, pp 598–604, DOI: [10.1016/S0076-6879\(55\)02263-5](https://doi.org/10.1016/S0076-6879(55)02263-5).
- (16) Bowen, W. J.; Kerwin, T. D. The kinetics of myokinase. I. Studies of the effects of salts and ph and of the state of equilibrium. *Archives of Biochemistry and Biophysics* **1954**, *49*, 149–159, DOI: [10.1016/0003-9861\(54\)90176-9](https://doi.org/10.1016/0003-9861(54)90176-9).
- (17) Brett, D.; Pospisil, H.; Valcárcel, J.; Reich, J.; Bork, P. Alternative splicing and genome complexity. *Nature genetics* **2002**, *30*, 29–30.
- (18) Amiri, M.; Conserva, F.; Panayiotou, C.; Karlsson, A.; Solaroli, N. The human adenylate kinase 9 is a nucleoside mono- and diphosphate kinase. *The International Journal of Biochemistry and Cell Biology* **2013**, *45*, 925–931, DOI: [10.1016/j.biocel.2013.02.004](https://doi.org/10.1016/j.biocel.2013.02.004).
- (19) Panayiotou, C.; Solaroli, N.; Xu, Y.; Johansson, M.; Karlsson, A. The characterization of human adenylate kinases 7 and 8 demonstrates differences in kinetic parameters and structural organization among the family of adenylate kinase isoenzymes. *Biochemical Journal* **2011**, *433*, 527–534.
- (20) Khoo, J. C.; Russell, P. J. Isoenzymes of adenylate kinase in human tissue. *Biochimica et Biophysica Acta (BBA)-Enzymology* **1972**, *268*, 98–101.
- (21) Wilson Jr, D.; POVEY, S.; HARRIS, H. Adenylate kinases in man: evidence for a third locus. *Annals of Human Genetics* **1976**, *39*, 305–313.

- (22) Matsuura, S.; Igarashi, M.; Tanizawa, Y.; Yamada, M.; Kishi, F.; Kajii, T.; Fujii, H.; Miwa, S.; Sakurai, M.; Nakazawa, A. Human adenylate kinase deficiency associated with hemolytic anemia: A single base substitution affecting solubility and catalytic activity of the cytosolic adenylate kinase. *Journal of Biological Chemistry* **1989**, *264*, 10148–10155, DOI: [10.1016/S0021-9258\(18\)81779-3](https://doi.org/10.1016/S0021-9258(18)81779-3).
- (23) Qualtieri, A.; Pedace, V.; Bisconte, M. G.; Bria, M.; Gulino, B.; Andreoli, V.; Brancati, C. Severe erythrocyte adenylate kinase deficiency due to homozygous A → G substitution at codon 164 of human AK1 gene associated with chronic haemolytic anaemia. *British Journal of Haematology* **1997**, *99*, 770–776, DOI: [10.1046/j.1365-2141.1997.4953299.x](https://doi.org/10.1046/j.1365-2141.1997.4953299.x).
- (24) Bianchi, P.; Zappa, M.; Bredi, E.; Vercellati, C.; Pelissero, G.; Barraco, F.; Zanella, A. A case of complete adenylate kinase deficiency due to a nonsense mutation in AK-1 gene (Arg 107→ Stop, CGA→ TGA) associated with chronic haemolytic anaemia. *British journal of haematology* **1999**, *105*, 75–79.
- (25) Pucar, D.; Janssen, E.; Dzeja, P. P.; Juranic, N.; Macura, S.; Wieringa, B.; Terzic, A. Compromised Energetics in the Adenylate Kinase AK1Gene Knockout Heart under Metabolic Stress. *Journal of Biological Chemistry* **2000**, *275*, 41424–41429, DOI: [10.1074/jbc.M007903200](https://doi.org/10.1074/jbc.M007903200).
- (26) Fukami-Kobayashi, K.; Nosaka, M.; Nakazawa, A.; Gō, M. Ancient divergence of long and short isoforms of adenylate kinase molecular evolution of the nucleoside monophosphate kinase family. *FEBS Letters* **1996**, *385*, 214–220, DOI: [10.1016/0014-5793\(96\)00367-5](https://doi.org/10.1016/0014-5793(96)00367-5).
- (27) Noma, T.; Song, S.; Yoon, Y.-S.; Tanaka, S.; Nakazawa, A. cDNA cloning and tissue-specific expression of the gene encoding human adenylate kinase isozyme 21The nucleotide sequence data reported in this paper have been submitted to the DDBJ, EMBL and GenBank nucleotide sequence databases under the accession numbers AB005621 (AK2A) and AB005622 (AK2B).1. *Biochimica et Biophysica Acta (BBA) - Gene Structure and Expression* **1998**, *1395*, 34–39, DOI: [10.1016/S0167-4781\(97\)00193-0](https://doi.org/10.1016/S0167-4781(97)00193-0).
- (28) Sorrentino, V.; Menzies, K. J.; Auwerx, J. Repairing mitochondrial dysfunction in disease. *Annual review of pharmacology and toxicology* **2018**, *58*, 353–389.
- (29) Lagresle-Peyrou, C.; Six, E. M.; Picard, C.; Rieux-Lauca, F.; Michel, V.; Ditadi, A.; Chappedelaine, C. D.-d.; Morillon, E.; Valensi, F.; Simon-Stoos, K. L., et

- al. Human adenylate kinase 2 deficiency causes a profound hematopoietic defect associated with sensorineural deafness. *Nature genetics* **2009**, *41*, 106–111.
- (30) Pannicke, U.; Hönig, M.; Hess, I.; Friesen, C.; Holzmann, K.; Rump, E.-M.; Barth, T. F.; Rojewski, M. T.; Schulz, A.; Boehm, T., et al. Reticular dysgenesis (aleukocytosis) is caused by mutations in the gene encoding mitochondrial adenylate kinase 2. *Nature genetics* **2009**, *41*, 101–105.
- (31) Lee, H.-J.; Pyo, J.-O.; Oh, Y.; Kim, H.-J.; Hong, S.-h.; Jeon, Y.-J.; Kim, H.; Cho, D.-H.; Woo, H.-N.; Song, S., et al. AK2 activates a novel apoptotic pathway through formation of a complex with FADD and caspase-10. *nature cell biology* **2007**, *9*, 1303–1310.
- (32) NOMA, T.; FUJISAWA, K.; YAMASHIRO, Y.; SHINOHARA, M.; NAKAZAWA, A.; GONDO, T.; ISHIHARA, T.; YOSHINOBU, K. Structure and expression of human mitochondrial adenylate kinase targeted to the mitochondrial matrix. *Biochemical journal* **2001**, *358*, 225–232.
- (33) Yoneda, T.; Sato, M.; Maeda, M.; Takagi, H. Identification of a novel adenylate kinase system in the brain: Cloning of the fourth adenylate kinase. *Molecular Brain Research* **1998**, *62*, 187–195, DOI: [10.1016/S0169-328X\(98\)00249-6](https://doi.org/10.1016/S0169-328X(98)00249-6).
- (34) Panayiotou, C.; Solaroli, N.; Johansson, M.; Karlsson, A. Evidence of an intact N-terminal translocation sequence of human mitochondrial adenylate kinase 4. *The International Journal of Biochemistry and Cell Biology* **2010**, *42*, 62–69, DOI: [10.1016/j.biocel.2009.09.007](https://doi.org/10.1016/j.biocel.2009.09.007).
- (35) Liu, R.; Ström, A.-L.; Zhai, J.; Gal, J.; Bao, S.; Gong, W.; Zhu, H. Enzymatically inactive adenylate kinase 4 interacts with mitochondrial ADP/ATP translocase. *The International Journal of Biochemistry and Cell Biology* **2009**, *41*, 1371–1380, DOI: [10.1016/j.biocel.2008.12.002](https://doi.org/10.1016/j.biocel.2008.12.002).
- (36) Solaroli, N.; Panayiotou, C.; Johansson, M.; Karlsson, A. Identification of two active functional domains of human adenylate kinase 5. *FEBS Letters* **2009**, *583*, 2872–2876, DOI: [10.1016/j.febslet.2009.07.047](https://doi.org/10.1016/j.febslet.2009.07.047).
- (37) Van Rompay, A. R.; Johansson, M.; Karlsson, A. Identification of a novel human adenylate kinase: cDNA cloning, expression analysis, chromosome localization and characterization of the recombinant protein. *European journal of biochemistry* **1999**, *261*, 509–517.

- (38) Ren, H.; Wang, L.; Bennett, M.; Liang, Y.; Zheng, X.; Lu, F.; Li, L.; Nan, J.; Luo, M.; Eriksson, S., et al. The crystal structure of human adenylate kinase 6: An adenylate kinase localized to the cell nucleus. *Proceedings of the National Academy of Sciences* **2005**, *102*, 303–308.
- (39) Drakou, C. E.; Malekkou, A.; Hayes, J. M.; Lederer, C. W.; Leonidas, D. D.; Oikonomakos, N. G.; Lamond, A. I.; Santama, N.; Zographos, S. E. hCINAP is an atypical mammalian nuclear adenylate kinase with an ATPase motif: Structural and functional studies. *Proteins: Structure, Function, and Bioinformatics* **2012**, *80*, 206–220, DOI: [10.1002/prot.23186](https://doi.org/10.1002/prot.23186).
- (40) Fernandez-Gonzalez, A.; Kourembanas, S.; Wyatt, T. A.; Mitsialis, S. A. Mutation of murine adenylate kinase 7 underlies a primary ciliary dyskinesia phenotype. *American journal of respiratory cell and molecular biology* **2009**, *40*, 305–313.
- (41) Badano, J. L.; Mitsuma, N.; Beales, P. L.; Katsanis, N. The Ciliopathies: An Emerging Class of Human Genetic Disorders. *Annual Review of Genomics and Human Genetics* **2006**, *7*, PMID: 16722803, 125–148, DOI: [10.1146/annurev.genom.7.080505.115610](https://doi.org/10.1146/annurev.genom.7.080505.115610).
- (42) Zariwala, M. A.; Knowles, M. R.; Omran, H. Genetic Defects in Ciliary Structure and Function. *Annual Review of Physiology* **2007**, *69*, PMID: 17059358, 423–450, DOI: [10.1146/annurev.physiol.69.040705.141301](https://doi.org/10.1146/annurev.physiol.69.040705.141301).
- (43) Simpson, K. J.; Selfors, L. M.; Bui, J.; Reynolds, A.; Leake, D.; Khvorova, A.; Brugge, J. S. Identification of genes that regulate epithelial cell migration using an siRNA screening approach. *Nature cell biology* **2008**, *10*, 1027–1038.
- (44) Dzeja, P.; Terzic, A. Adenylate kinase and AMP signaling networks: metabolic monitoring, signal communication and body energy sensing. *International journal of molecular sciences* **2009**, *10*, 1729–1772.
- (45) Boissan, M.; Dabernat, S.; Peuchant, E.; Schlattner, U.; Lascu, I.; Lacombe, M.-L. The mammalian Nm23/NDPK family: from metastasis control to cilia movement. *Molecular and cellular biochemistry* **2009**, *329*, 51–62, DOI: [10.1007/s11010-009-0120-7](https://doi.org/10.1007/s11010-009-0120-7).
- (46) Nelson, D.; Nelson, R.; Cox, M., *Lehninger Principles of Biochemistry, Fourth Edition and Lecture Notebook*; W.H. Freeman: 2004.

- (47) Barraud, P.; Amrein, L.; Dobremez, E.; Dabernat, S.; Masse, K.; Larou, M.; Daniel, J.-Y.; Landry, M. Differential expression of nm23 genes in adult mouse dorsal root ganglia. *Journal of Comparative Neurology* **2002**, *444*, 306–323, DOI: [10.1002/cne.10150](https://doi.org/10.1002/cne.10150).
- (48) Wang, L. The role of Ureaplasma nucleoside monophosphate kinases in the synthesis of nucleoside triphosphates. *The FEBS Journal* **2007**, *274*, 1983–1990, DOI: [10.1111/j.1742-4658.2007.05742.x](https://doi.org/10.1111/j.1742-4658.2007.05742.x).
- (49) Lu, Q.; Inouye, M. Adenylate kinase complements nucleoside diphosphate kinase deficiency in nucleotide metabolism. *Proceedings of the National Academy of Sciences* **1996**, *93*, 5720–5725, DOI: [10.1073/pnas.93.12.5720](https://doi.org/10.1073/pnas.93.12.5720).
- (50) Lodish, H.; Berk, A.; Zipursky, S.; Baltimore, D.; Darnell, J.; Matsudaira, P., *Molecular Cell Biology*; Cd-Rom; W.H. Freeman: 2000.
- (51) Berg, J.; Tymoczko, J.; Stryer, L., *Biochemistry, Fifth Edition*; [NCBI bookshelf]; W. H. Freeman: 2002.
- (52) Cattaneo, M.; Gachet, C. ADP receptors and clinical bleeding disorders. *Arteriosclerosis, thrombosis, and vascular biology* **1999**, *19*, 2281–2285.
- (53) Reem, G. H. et al. De novo purine biosynthesis by two pathways in Burkitt lymphoma cells and in human spleen. *The Journal of Clinical Investigation* **1972**, *51*, 1058–1062.
- (54) Reichard, P. Interactions between deoxyribonucleotide and DNA synthesis. *Annual review of biochemistry* **1988**, *57*, 349–374.
- (55) Arnér, E. S.; Eriksson, S. Mammalian deoxyribonucleoside kinases. *Pharmacology and therapeutics* **1995**, *67*, 155–186.
- (56) Galmarini, C. M.; Mackey, J. R.; Dumontet, C. Nucleoside analogues and nucleobases in cancer treatment. *The Lancet Oncology* **2002**, *3*, 415–424, DOI: [10.1016/S1470-2045\(02\)00788-X](https://doi.org/10.1016/S1470-2045(02)00788-X).
- (57) Van Rompay, A. R.; Johansson, M.; Karlsson, A. Phosphorylation of nucleosides and nucleoside analogs by mammalian nucleoside monophosphate kinases. *Pharmacology and Therapeutics* **2000**, *87*, 189–198, DOI: [10.1016/S0163-7258\(00\)00048-6](https://doi.org/10.1016/S0163-7258(00)00048-6).

- (58) Dimmock, D. P.; Dunn, J. K.; Feigenbaum, A.; Rupar, A.; Horvath, R.; Freisinger, P.; Mousson de Camaret, B.; Wong, L.-J.; Scaglia, F. Abnormal neurological features predict poor survival and should preclude liver transplantation in patients with deoxyguanosine kinase deficiency. *Liver Transplantation* **2008**, *14*, 1480–1485, DOI: [10.1002/lt.21556](https://doi.org/10.1002/lt.21556).
- (59) Lindemalm, S.; Liliemark, J.; Juliusson, G.; Larsson, R.; Albertoni, F. Cytotoxicity and pharmacokinetics of cladribine metabolite, 2-chloroadenine in patients with leukemia. *Cancer Letters* **2004**, *210*, 171–177, DOI: [10.1016/j.canlet.2004.03.007](https://doi.org/10.1016/j.canlet.2004.03.007).
- (60) Lewis, W.; Dalakas, M. C. Mitochondrial toxicity of antiviral drugs. *Nature medicine* **1995**, *1*, 417–422.
- (61) Leist, T. P.; Weissert, R. Cladribine: mode of action and implications for treatment of multiple sclerosis. *Clinical neuropharmacology* **2011**, *34*, 28–35.
- (62) Arnér, E. S. J. On the Phosphorylation of 2-chlorodeoxy-adenosine (CdA) and Its Correlation with Clinical Response in Leukemia Treatment. *Leukemia and Lymphoma* **1996**, *21*, PMID: 8726403, 225–231, DOI: [10.3109/10428199209067604](https://doi.org/10.3109/10428199209067604).
- (63) Galmarini, C.; Mackey, J.; Dumontet, C. Nucleoside analogues: mechanisms of drug resistance and reversal strategies. *Leukemia* **2001**, *15*, 875–890, DOI: [10.1038/sj.leu.2402114](https://doi.org/10.1038/sj.leu.2402114).
- (64) Lukenbill, J.; Kalaycio, M. Fludarabine: A review of the clear benefits and potential harms. *Leukemia Research* **2013**, *37*, 986–994, DOI: [10.1016/j.leukres.2013.05.004](https://doi.org/10.1016/j.leukres.2013.05.004).
- (65) Noble, S.; Goa, K. L. Gemcitabine: a review of its pharmacology and clinical potential in non-small cell lung cancer and pancreatic cancer. *Drugs* **1997**, *54*, 447–472, DOI: [10.2165/00003495-199754030-00009](https://doi.org/10.2165/00003495-199754030-00009).