

Chapter 2

Adenosine Metabolism, Adenosine Kinase, and Evolution

Jaoek Park and Radhey S. Gupta

Abstract The enzyme adenosine kinase (AdK, ADK, or AK) plays an important role in regulating the intracellular as well as extracellular concentrations of adenosine and hence its diverse physiological actions. In view of the enormous pharmacological potential of adenosine, there has been much interest in studying adenosine kinase over the past few decades. This chapter summarizes the wealth of information that has accumulated concerning its structure and function. The aspects that are reviewed include the enzymological aspects of ADK including its reaction mechanism and ionic requirement; insights provided by the crystal structure of the enzyme; a brief overview of work on identification and development of ADK inhibitors; novel aspects of the ADK gene structure; tissue distribution and subcellular localization of the two ADK isoforms; novel information provided by mammalian cells harboring mutations of ADK; and lastly the evolutionary relationship of ADK to other related proteins. Despite enormous progress several important gaps exist in our knowledge regarding ADK, particularly concerning the cellular functions of the two isoforms and how their relative amounts in different tissues are regulated, that need to be understood in order to fully realize the therapeutic potential of increased local concentration of adenosine by modulation of this key enzyme.

Keywords Adenosine kinase • Long and short isoforms • Protein structure • AK gene structure • Chinese hamster cell mutants • Differential localization of AdK isoforms • Pentavalent ion dependency

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2.1 Adenosine Kinase: Function and Role in Adenosine Metabolism

2.1.1 Adenosine

The purine nucleoside adenosine with neuromodulating properties produces a broad range of physiological responses in mammalian tissues via interaction with adenosine receptors. Much interest in adenosine metabolism has arisen from the discovery that adenosine receptor-mediated signals are involved in the protection of cells and tissues undergoing ischemic stress. Under normal oxygenated conditions, the rates of ATP catabolism and anabolism are equal, and this maintains stable cytosolic concentrations of both ATP and ADP (5–10 mM and 40–60 μ M in the heart, respectively) (Gard et al. 1985; Decking et al. 1997). This high ratio of [ATP] and [ADP] is accompanied by a low level of AMP, whose increase in concentration indicates a threat to the ATP supply. Under conditions of tissue stress or trauma, such as ischemia or hypoxia, the oxygen supply is inadequate for oxidative phosphorylation of ADP to regenerate ATP, and the [ATP]–[ADP] ratio is compromised (Headrick and Willis 1990). The increasing concentration of ADP leads to adenylate kinase activity (i.e., $2 \text{ ADP} \leftrightarrow \text{ATP} + \text{AMP}$) in the direction of AMP formation to maintain a stable [ADP]–[AMP] ratio (Ballard 1970). There is a subsequent net increase in the concentration of AMP (Headrick and Willis 1990), which in turn is hydrolyzed to adenosine (Decking et al. 1997; Gustafson and Kroll 1998; Bak and Ingwall 1998). Due to the increased cytosolic concentration, adenosine is released into the extracellular space, where it can bind to adenosine receptors (see Fig. 2.1).

Adenosine receptor-mediated responses (receptor subtypes: A_1 , A_{2A} , A_{2B} , and A_3) can have profound protective effects throughout the entire human body, including the nervous, cardiovascular, gastrointestinal, renal, muscular, and immune systems (Mubagwa and Flameng 2001; Stone et al. 2009; Wojcik et al. 2010; Zylka 2011). For this reason, adenosine has been variously called as “signal of life,” “retaliatory metabolite,” and “body’s natural defense” (Engler 1991; Mullane and Bullough 1995; Cohen and Downey 2008; Boison 2008a). Naturally, there has been an enormous research interest in studying the therapeutic potential of adenosine. The list of prospective areas for its medical application is expanding rapidly; because these topics are covered in other chapters, only a brief mention of them is made here. Adenosine has been shown to provide protection against ischemia–reperfusion injuries in the heart (Mubagwa and Flameng 2001), brain and spinal cord (Phillis and Goshgarian 2001; Williams-Karnesky and Stenzel-Poore 2009), and kidney (Bauerle et al. 2011). It also exhibits potent pain-relieving effects in diverse preclinical models of chronic pain (Zylka 2011). Adenosine is a powerful anticonvulsant against epilepsy (Boison and Stewart 2009), and it participates in the pathology of several neuronal and neurodegenerative disorders such as schizophrenia, Alzheimer’s disease, Huntington’s disease, amyotrophic lateral sclerosis, and multiple sclerosis (Boison 2008a; Stone et al. 2009; Amadio et al. 2011; Boison et al. 2011). Recent work by

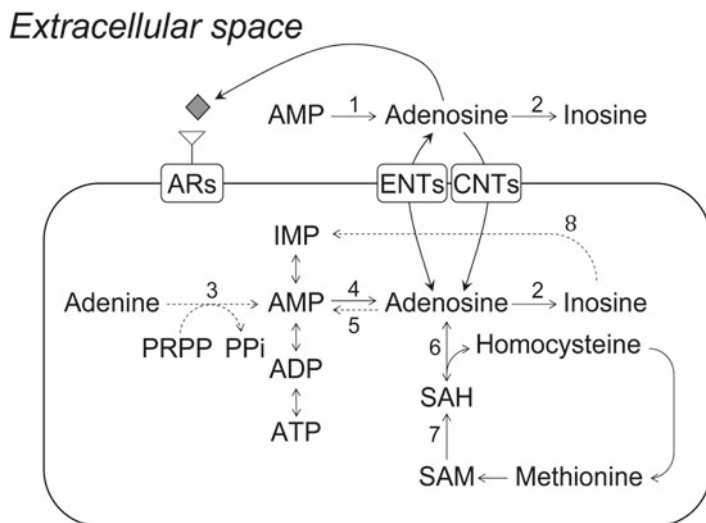


Fig. 2.1 Adenosine metabolism, transport, and receptor binding: a general overview. Enzymes: 1, ecto-5'-nucleotidase (5'-NT); 2, adenosine deaminase (ADA); 3, adenine phosphoribosyltransferase (APRTase); 4, 5'-nucleotidase (cN-I); 5, adenosine kinase (ADK); 6, S-adenosyl-L-homocysteine hydrolase; 7, various methyl-transferases and 8, inosine-guanosine kinase. AR adenosine receptor, ENT equilibrative nucleoside transporter, CNT concentrative nucleoside transporter, PRPP 5-phosphoribose-1-pyrophosphate, PPi inorganic pyrophosphate, SAH S-adenosyl-L-homocysteine, SAM S-adenosyl-L-methionine. The salvage pathway reactions are shown as dotted arrows. Modified from Park and Gupta 2008

Boison's group establishes key roles for adenosine and ADK in the development of epilepsy in a mouse model (Boison 2008b, 2010). Their work has led to "Adenosine Kinase hypothesis of Epileptogenesis," according to which the increased production of ADK by astrocytes in astrogliosis, leads to a lowering of adenosine concentration in critical brain regions, thereby resulting in the loss of inhibition of neuronal excitation leading to epileptic seizure (Boison 2008b, 2010). Because adenosine augmentation by inhibition of ADK, or by other means such as implantation of encapsulated cells engineered to release adenosine, provided protection from epileptic seizure, it establishes the usefulness of these modalities for development of novel therapies for treatment of epilepsy (Boison and Stewart 2009; Boison 2009a, b, 2011; Theofilas et al. 2011).

2.1.1.1 Adenosine Metabolizing Enzymes

As adenosine initiates signaling cascades that can produce such intense and broad downstream effects, its cellular concentration needs to be tightly regulated. The cytosolic concentration of adenosine under normoxic conditions is estimated to range between 0.01 and 0.1 μM (Kroll et al. 1992; Deussen et al. 1999). Interstitial

concentrations of adenosine during hypoxic episodes, on the other hand, have been found as high as 30 μM within the myocardium (Manthei et al. 1998), 30 μM in the brain (Latini et al. 1999), and 100 μM in the spinal cord (McAdoo et al. 2000). The enzymes responsible for lowering adenosine concentration are adenosine kinase (ADK) and adenosine deaminase (ADA), which catalyze the phosphorylation and deamination of adenosine to produce AMP and inosine, respectively (Fig. 2.1). The K_m of ADA for adenosine is in the range of 25–150 μM (depending upon the iso-form) (Ford et al. 2000; Singh and Sharma 2000), whereas that of ADK is approximately 1 μM (De Jong 1977; Drabikowska et al. 1985). Because the K_m of ADK for adenosine is much lower than that of ADA, ADK is thought to be the principal enzyme responsible for regulating the level of adenosine under physiological conditions. In proof of this concept, inhibition of ADK, but not of ADA, resulted in increased adenosine concentration and its subsequent release into the interstitial space in rat brain slices (Pak et al. 1994).

2.1.1.2 Adenosine Producing Enzymes

While ADK and ADA are the enzymes responsible for the removal of cellular adenosine, there are three enzymes whose immediate actions result in the formation of adenosine. The most significant source of adenosine during enhanced oxygen demand or metabolic load, quantitatively, is the AMP-selective cytosolic 5'-nucleotidase (cN-I, Fig. 2.1), which hydrolyzes AMP into adenosine and inorganic phosphate. Some studies have suggested that this enzyme is responsible for more than 70 % of adenosine production in mammalian heart cells (Darvish et al. 1996; Garvey and Prus 1999). Another enzyme responsible for the production of adenosine is ecto-5'-nucleotidase (5'-NT, Fig. 2.1), a glycoprotein found on the plasma membrane of eukaryotic cells (Misumi et al. 1990). Although this enzyme carries out the same reaction as cN-I, its sequence bears no similarity to that of the latter enzyme, representing a case of convergent evolution (Sala-Newby et al. 1999). Interestingly, 5'-NT is inhibited by both ADP and ATP (Sullivan and Alpers 1971; Naito and Lowenstein 1985), whereas cN-I is allosterically activated by ADP but not by ATP (Yamazaki et al. 1991; Darvish and Metting 1993). The end result is probably that the relative contribution of each enzyme to the total adenosine pool varies as a function of its tissue-specific expression, as well as the cellular energy status. In addition to these two enzymes, adenosine is also produced in heart by hydrolysis of *S*-adenosylhomocysteine (SAH) via SAH-hydrolase (Fig. 2.1). Although this later mechanism is responsible for the production of majority of basal adenosine in heart under normoxia, its role in other tissues such as brain seems to be quite limited (Pascual et al. 2005; Dulla et al. 2005; Frenguelli et al. 2007). The reaction catalyzed by SAH-hydrolase is reversible, and the direction of its activity is dependent on the local concentrations of adenosine and homocysteine (Loncar et al. 1997). The enzyme, however, generally favors the hydrolysis of SAH, as both adenosine and homocysteine are rapidly metabolized under normal conditions (Deussen et al. 1988).

2.1.1.3 Adenosine Transport

In addition to its role as a signaling molecule that communicates intracellular metabolic events to receptors on the cell surface, adenosine has another important cellular function: a building block for ATP, and ultimately nucleic acids, via the salvage pathway. Adenosine recycling is common and important, as *de novo* nucleotide synthesis is energetically expensive and highly tissue-specific. Both adenosine signaling and adenosine salvage require efficient transport of the molecule across the plasma membrane (Fig. 2.1). Since adenosine is hydrophilic, this is mediated by specialized membrane-associated proteins. Two gene families have been identified to code for such proteins in mammals: solute carrier families 28 and 29 (SLC28 and SLC29) (Molina-Arcas et al. 2009). SLC28 encodes the concentrative nucleoside transporters, CNT1, CNT2, and CNT3, which carry out unidirectional (inwardly directed), sodium-coupled, energy-dependent active transport (Gray et al. 2004). SLC29 encodes the equilibrative nucleoside transporters, ENT1, ENT2, ENT3, and ENT4, which mediate bidirectional, sodium-independent facilitated diffusion (Baldwin et al. 2004). ENTs are considered as ubiquitous transporters, expressed in a wide range of tissues including the vascular endothelium, skeletal muscles, heart, brain, liver, kidney, and placenta (Griffiths et al. 1997; Pennycooke et al. 2001; Baldwin et al. 2005; Barnes et al. 2006). CNTs, on the other hand, have been found in specialized epithelial cells in the small intestine, kidney, and liver (Felipe et al. 1998; Valdes et al. 2000; Pennycooke et al. 2001), as well as in immune cells (Pennycooke et al. 2001; Soler et al. 2001; Minuesa et al. 2008) and the brain (Anderson et al. 1996; Ritzel et al. 2001; Guillen-Gomez et al. 2004).

Equilibrative Nucleoside Transporters

While all of the four ENTs can mediate the influx and efflux of adenosine, their capacity to transport adenosine and other nucleosides varies. Human ENT1 and ENT2, for example, display a broad selectivity of substrates, transporting most purines and pyrimidines (Crawford et al. 1998; Yao et al. 2002). Both transporters show the highest affinity for adenosine out of all the nucleoside substrates, with the apparent K_m values of 40 and 100 μM , respectively (Molina-Arcas et al. 2009). ENT2, but not ENT1, is able to transport various nucleobases as well (Osses et al. 1996). ENT3, on the other hand, can also transport a wide range of purine and pyrimidine nucleosides but shows relatively low affinity for adenosine ($K_m \approx 2 \text{ mM}$ for the human protein) (Baldwin et al. 2005). This protein contains two endosomal/lysosomal targeting motifs in its N-terminus (Hyde et al. 2001) and shows the optimum pH value of 5.5 (Baldwin et al. 2005), which reflects its location in acidic, intracellular compartments (Molina-Arcas et al. 2009). ENT4, in contrast to the other ENTs, transports adenosine (Baldwin et al. 2004) and serotonin (Engel et al. 2004) but no other nucleosides. Its adenosine transport activity is also optimal at acidic pH (apparent K_m values of 0.78 and 0.13 mM for the human and mouse forms, respectively, at pH 5.5) but absent at pH 7.5 (Barnes et al. 2006). ENT4's abundance

in the heart, particularly in the plasma membranes of ventricular myocytes and vascular endothelial cells, suggests its pH optimum is related to the acidotic conditions associated with ischemia (Barnes et al. 2006).

Concentrative Nucleotide Transporters

In comparison to ENTs, CNTs show higher affinity as well as selectivity for their nucleoside substrates (Gray et al. 2004). CNT1 is a pyrimidine-preferring transporter and cannot transport adenosine, which nevertheless binds to the translocation site of the protein with high affinity ($K_d = 14 \mu\text{M}$ for the human protein) (Larrayoz et al. 2004). CNT2 is a purine-preferring transporter, although it can also transport uridine (Gray et al. 2004). CNT3, on the other hand, shows broader substrate specificity and translocates both purine and pyrimidine nucleosides (Ritzel et al. 2001). The apparent K_m values of human CNT2 and CNT3 for adenosine have shown to be 8 and 15 μM , respectively (Molina-Arcas et al. 2009). In addition to their substrate specificity and tissue distribution, these transporters also differ in their cotransport requirement. CNT1 and CNT2, for example, transport one sodium ion per nucleoside (Ritzel et al. 1998; Smith et al. 2004), while CNT3 transport two sodium ions (Ritzel et al. 2001). Also, CNT3 shows pH-dependent nucleoside transport, as it can cotransport H^+ with nucleosides as well (in a 1:1 stoichiometry) (Smith et al. 2005). CNT1 and CNT2, on the contrary, are strictly Na^+ -dependent. As mammalian tissues express several nucleoside transporters in a single cell type, often combining both the concentrative and equilibrative proteins, the regulation of extracellular adenosine is a complex process. Termination of adenosine signaling is a result of concerted work by these nucleoside transporters as well as the adenosine-metabolizing enzymes, ADK and ADA.

2.1.2 Adenosine Kinase

As one of the most ubiquitous and abundant nucleoside kinases known (Krenitsky et al. 1974; Snyder and Lukey 1982), ADK (EC 2.7.1.20) is the first enzyme in the metabolic pathway of adenosine salvage. ADK functions as an important regulator of extracellular adenosine, as clearly demonstrated in animal models of epilepsy and ischemia (Pignataro et al. 2007, 2008; Li et al. 2008). ADK in addition carries out an essential role in facilitating cellular methylation reactions, and its deficiency in transgenic $\text{ADK}^{-/-}$ mice leads to development of acute neonatal hepatic steatosis, which causes early postnatal mortality (Boison et al. 2002). ADK is also responsible for the phosphorylation of other nucleosides and their analogs into the corresponding monophosphates, which include antimetabolites that have profound biological effects (Gupta 1989). After its first isolation from yeast and mammalian tissues 60 years ago (CAPUTTO 1951), an extensive amount of work has been carried out on many different aspects of this important enzyme.

2.1.2.1 Enzymological Aspects of Adenosine Kinase

One of the unique characteristics of ADK reaction (i.e., Adenosine + ATP $\rightarrow$ AMP + ADP) is that the phosphoryl accepting substrate adenosine shares the same structural motif with the phosphoryl donor ATP. As a result, both adenosine and its phosphorylated product AMP bind to the ATP site (Pelicano et al. 1997), resulting in enzyme inhibition. As this complicates the inhibition pattern of the enzyme, it has been difficult to interpret the kinetic data and come to a consensus in regards to ADK's reaction mechanism. Chang et al. (1983) suggested a two-site ping-pong mechanism, while several others proposed ordered Bi Bi mechanisms (Henderson et al. 1972; Palella et al. 1980; Mimouni et al. 1994a). The order of substrate binding and product release suggested in each of these studies also differed from one another. The information obtained from the crystal structures of human (Mathews et al. 1998) and *Toxoplasma gondii* (Schumacher et al. 2000) ADK, on the other hand, allowed clear understanding of the reaction mechanism: an ordered Bi Bi mechanism, in which adenosine is the first substrate to bind, and AMP is the last product to dissociate.

Another interesting aspect of ADK, which also complicates its enzymological characterization, is its adenosine-AMP exchange activity. In characterizing ADK from rat liver (Mimouni et al. 1994a, b) and Chinese hamster cells (Hao and Gupta 1996; Gupta 1996), it was discovered that the enzyme could transfer the phosphate from AMP to radioactively labeled adenosine in the absence of ATP. This at first suggested a classical ping-pong mechanism, but formation of a phosphoryl-enzyme intermediate could not be demonstrated. The exchange reaction showed complete dependence on the presence of ADP and Mg^{2+} (Mimouni et al. 1994a; Gupta 1996), and thus could be explained with the backward reaction of ADK. In the exchange reaction, the enzyme first carries out the backward reaction, in which AMP and ADP are converted to adenosine and ATP. Accumulation of ATP then allows the forward reaction, in which both the labeled and nonlabeled adenosine is phosphorylated without discrimination. Overall, therefore, ADP functions as a carrier compound that shuttles a phosphate group from one adenosine to another. This finding also corroborates a Bi Bi reaction mechanism for ADK.

Magnesium Requirement

With respect to the phosphate-donating substrate, ATP and GTP are the preferred nucleotides for ADK, although other nucleotides can also participate in the catalytic reaction with lower efficiency (Miller et al. 1979, 1982; Chang et al. 1983; Rotllan and Miras Portugal 1985). However, the true phosphate-donating substrate of ADK, like most kinases, is the complex of a nucleotide and a divalent metal ion, typically magnesium. In a reaction mixture, ATP exists in several states of ionization depending on the pH of the solution. Mg^{2+} binds to ATP^{4-} , the fully dissociated species of ATP, and forms $MgATP^{2-}$, which then binds to ADK. In support of this concept, absence of magnesium in the reaction resulted in lack of enzyme activity in human ADK, while in the presence of magnesium, optimal activity was observed at pH

values where ATP and the divalent cation exist primarily in the chelated form (Palella et al. 1980). The magnesium ion is thought to neutralize the negative charges on the phosphate groups of the nucleotide for its binding to the enzyme (Mildvan 1987).

The effect of free magnesium on ADK should also be noted. Excess magnesium in a reaction results in the saturation of available ATP^{4-} , and thus, presence of free Mg^{2+} . Interestingly, ADK activity increases as the level of free Mg^{2+} in the reaction increases up to an optimal concentration, after which point additional free Mg^{2+} leads to inhibition of the enzyme (Palella et al. 1980; Rotllan and Miras Portugal 1985; Maj et al. 2002a). A similar free magnesium requirement for optimal catalysis has also been observed in other ATP-utilizing enzymes, including 6-phosphofructo-2-kinase (Parducci et al. 2006), phosphoribosylpyrophosphate synthetase (Arnvig et al. 1990), pyruvate kinase (Baek and Nowak 1982), and phosphoenolpyruvate kinase (Lee et al. 1981). The free, catalytic magnesium ion is thought to bind at the active site and induce the transition state of the reaction by increasing the electrophilicity of the γ -phosphorous atom of the nucleotide via its interaction with the oxygen atoms (Parducci et al. 2006). In addition, this ion may induce tighter binding and/or proper orientation of the substrate's functional groups (Rivas-Pardo et al. 2011).

Substrate Specificity

As for the phosphate-accepting, nucleoside substrate, ADK shows remarkably broad specificity. Alteration of the ribosyl moiety of adenosine is well tolerated, although this decreases substrate efficiency by two- to fivefold (Bennett and Hill 1975). One important structural requirement is that the nucleoside must have a 2'-hydroxyl group, and this hydroxyl group should be in the *trans* conformation. If these conditions are met, the 3'-hydroxyl and 4'-hydroxyl-methyl groups may be in either *trans* or *cis* conformation. Substitution or addition of functional groups in the purine base moiety can also be well accepted. In fact, compounds such as 8-azaadenosine, show even higher phosphorylation efficiency than the natural substrate adenosine (Bennett and Hill 1975). The nucleosides and their analogs that are efficiently phosphorylated by ADK generally differ from adenosine at the 2-, 6-, 7-, 8-, and 9-substituent positions (Lindberg et al. 1967; Bennett and Hill 1975; Miller et al. 1979).

One of the direct consequences of ADK's broad selectivity for the nucleoside substrate is that the enzyme can activate a large number of antimetabolites. Adenosine analogs, such as fludarabine, are easily transported into the cell and after phosphorylation produce cytotoxic effects by interfering with DNA synthesis (Parker et al. 2004). A promising new area of application for these antimetabolites, in addition to the current anticancer and antiviral applications, is antiparasitic chemotherapy (Datta et al. 2008). Nucleoside kinases are important in parasitic protozoa, such as *Leishmania*, *Trypanosoma*, and *Toxoplasma*, due to their absolute reliance on the salvage of preformed purines from their hosts. ADK in these organisms shows profoundly different substrate specificity compared to the human enzyme (Luscher et al. 2007; Al Safarjalani et al. 2008; Cassera et al. 2011) and thus may allow selective activation of adenosine antimetabolites in the invading parasite but not in the host cells.

Regulation by Inorganic Phosphate

In addition to divalent metal ions, the activity of ADK is also affected by inorganic phosphate, whose precise mechanism of action is not fully understood. This property of ADK, which is referred to as phosphate dependency, was first demonstrated with the Chinese hamster enzyme (Hao and Gupta 1996). In this study, the catalytic activity of ADK was shown to be almost completely dependent on the presence of inorganic phosphate. The addition of inorganic phosphate served to increase the maximum velocity of ADK, as well as to decrease the K_m for its substrate, adenosine. Interestingly, other phosphate analogs, such as arsenate and vanadate, were able to substitute for inorganic phosphate as activators of ADK (Hao and Gupta 1996). Kinetic studies have shown that these ions also function to increase the V_{max} of ADK and to decrease the K_m for adenosine. On the contrary, arsenate, vanadate, and phosphate showed little effect on the K_m for ATP. ADK isolated from different sources, such as human, cow, spinach, yeast, and a protist, *Leishmania donovani*, have all shown phosphate dependency, indicating that this is a conserved property of the enzyme (Maj et al. 2000, 2002; Park et al. 2006).

In addition to ADK, there are many other enzymes which have shown phosphate dependency, including ribokinase (EC 2.7.1.15) (Maj and Gupta 2001), 6-phosphofructo-1-kinase (EC 2.7.1.11) (Hofer et al. 1982), 6-phosphofructo-2-kinase (EC 2.7.1.105) (Laloux et al. 1985), and phosphoribosylpyrophosphate synthetase (EC 2.7.6.1) (Switzer 1969). Like ADK, these enzymes catalyze the transfer of phosphate from ATP to a sugar-derived substrate. Importantly, these enzymes are all involved in the production of essential, high-energy metabolites. It is not surprising that the activities of these enzymes are regulated by inorganic phosphate, whose concentration can function as an index of the cellular energy-status. The concentration of inorganic phosphate measured in guinea pig hearts, for example, was 1.7, 7.1 and 13.9 mM under normal, mildly hypoxic, and severely hypoxic conditions, respectively (Gorman et al. 1997). Inorganic phosphate results in half maximal velocity of ADK at about 2 mM, and maximal velocity at about 10 mM concentration in vitro (Park et al. 2004, 2006). The physiological level of inorganic phosphate, therefore, allows a full range of control for ADK.

The activation of ADK by phosphate has led to an examination of many other phosphorylated compounds and their analogs (Park et al. 2004, 2006). This study has identified a number of additional compounds that can activate mammalian ADK. Kinetic experiments showed that these compounds act like inorganic phosphate, resulting in increased V_{max} of the enzyme and decreased K_m for adenosine. Most of the activators identified are common and important high-energy cellular metabolites, such as acetyl phosphate, carbamoyl phosphate, and phosphoenolpyruvate, and may have a physiologically relevant role as regulators of the phosphate-dependent enzymes. Interestingly, a number of compounds that showed structural homology to the activators instead inhibited ADK in the same study (Park et al. 2004, 2006). The fact that these activators and inhibitors show structural homology indicates that there is a structural basis for phosphate dependency. This thought is further supported by the observation that the inhibitor compounds inhibit ADK in a competitive manner

with respect to the activating inorganic phosphate. The hypothesis is that both the activators and inhibitors, sharing similar structure motifs, can bind to the phosphate regulatory site on ADK. Although bound to the regulatory site, however, the inhibitor compounds cannot promote enzyme catalysis due to the differences in their electrochemical properties (Park and Gupta 2008). The inhibitors thus elicit enzyme inhibition by sequestering the regulatory (or activation) site, which phosphate or other activator compounds need to occupy for full enzyme activity.

In this light, it is very interesting that crystal structures of ribokinase, an enzyme evolutionarily related to ADK with a remarkable structural homology, were determined with a bound phosphate in the active site (Sigrell et al. 1998; 1999). This phosphate makes close contacts with the residues Asn187 and Glu190, forming direct and indirect hydrogen interactions. The asparagine and glutamic acid residues at these positions are highly conserved in the ribokinase family, to which both ADK and ribokinase belong, and identify a new sequence motif, NXXE (Maj et al. 2002). Site-directed mutations of these residues (Glu242 and N239) have been introduced in Chinese hamster ADK (Maj et al. 2002). Some of the mutants were completely inactivated as a result, but the ones with remaining activity (E242D and N239Q mutants) showed a significantly altered phosphate requirement, demonstrating the importance of the NXXE motif in the phosphate activation of ADK. These mutants also showed decreased affinity for free Mg^{2+} , indicating that the phosphate regulation of ADK activity is closely related to the catalytic magnesium ion. Mutation of the corresponding Glu190 residue in *E. coli* 6-phosphofructo-2-kinase, another member of the ribokinase family that show phosphate-dependency, also resulted in a protein with an altered Mg^{2+} requirement (Parducci et al. 2006). These results suggest the NXXE motif as the site of phosphate-binding, which in turn is required for proper binding of the catalytic Mg^{2+} at the active site.

Crystallographic Structure

Further insights into ADK's mechanism of action have been provided with the enzyme's crystal structures. The human enzyme was the first to be crystallized and characterized (Mathews et al. 1998). ADK exists as a monomer (Fig. 2.2a shows the overall structure of human ADK), which comprises two distinct domains, a $\alpha\beta\alpha$ three layer sandwich domain and a smaller lid-like domain. The larger domain is formed by a central nine-stranded β -sheet which is flanked by ten α -helices and provides most of the specific binding interactions for the two substrates, adenosine and ATP. The smaller domain contains a five-stranded mixed β -sheet flanked by two α -helices on the side exposed to the solvent and acts as a lid over the active site. Four peptide segments connect these domains, and the substrate adenosine binds in the cleft formed between the two domains. The nucleotide binding site, on the other hand, is located in a groove in the $\alpha\beta\alpha$ domain and is not entirely covered by the smaller lid domain. Important residues involved in the binding of adenosine include Leu16, Leu40, Leu134, Ala136, Leu138, Phe170, and Phe201, which form the hydrophobic pocket for the substrate and provide stacking interaction with its adenine base. The residues

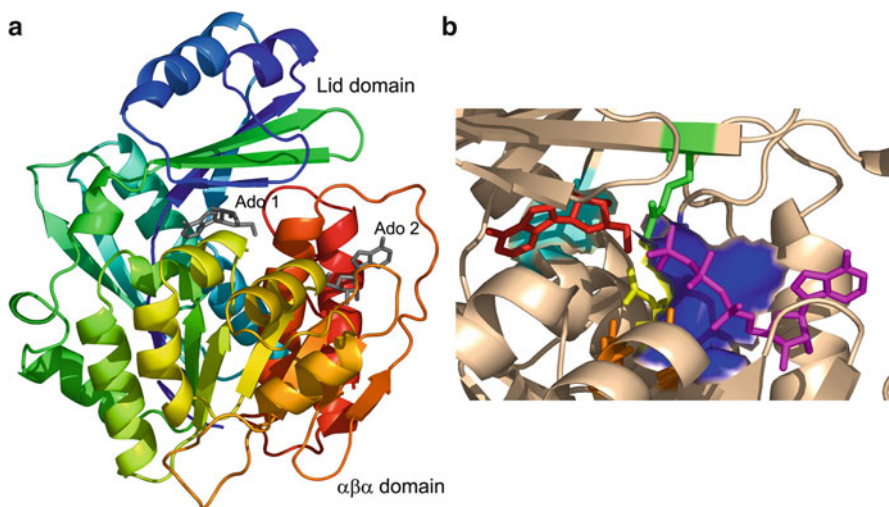


Fig. 2.2 Crystal structures of adenosine kinase. (a) A cartoon representation of human adenosine kinase (PDB code: 1BX4) with two bound adenosine molecules. The enzyme is shown in a *rainbow color* scheme (blue at the N-terminus and red at the C-terminus), whereas the adenosine molecules (Ado 1 and Ado 2) are depicted as *sticks in gray*. The second adenosine ligand (Ado 2) is bound at the ATP-binding site. (b) A close-up view of *Toxoplasma gondii* adenosine kinase (PDB code: 1LII) active site. Bound adenosine and AMP-PCP are shown in red and magenta, respectively. A number of other important structural features are also shown in this representation: i) the surface of the anion hole (blue); ii) the surface of the GG switch (cyan); Asn223 and Glu226 of the NXXE motif (orange); the catalytic base Asp318 (yellow); and Arg136 of the lid domain which shows a close contact with the bound nucleotide (green)

involved in ATP-binding, on the other hand, include Asn196, Asn223, Glu226, Thr265, Gly267, Val284, and Ile292, which provide fewer hydrophobic interactions (hence higher K_m for the nucleotide) compared to those in the adenosine site.

Additional information on ADK structure and function became available when the crystal structures of the enzyme from *T. gondii*, a parasitic protozoan, were determined (Schumacher et al. 2000). In this study, the structures of both apo enzyme and the substrate-bound forms were solved, and their comparison revealed drastic conformational differences between them. The most striking difference is the 30° hinge bending caused by the binding of adenosine, which brings the large and small domains together. This conformational change can be conveniently described as an opening and closing of the lid domain, if the $\alpha\beta\alpha$ domain is taken as a point of reference. In this case, the apo structure takes the “open” conformation, exposing the adenosine-binding site to the surrounding solvent environment. The substrate-bound structure, on the contrary, takes the “closed” conformation, in which the bound adenosine is almost completely buried under the lid domain. This conformational change is likely brought about by residues Gly68 and Gly69, called GG switch (Fig. 2.2b), which undergo large torsional changes as they interact with adenosine. Subsequent ATP (AMP-PCP in the crystal structure) binding to the adenosine–enzyme complex induces a less dramatic but nonetheless important

conformational change: an anion hole is created around the β - and γ -phosphates of the ATP (Fig. 2.2b). These phosphates also interact with the residue Arg136 (Fig. 2.2b), which moves into the active site by 13.7 Å upon adenosine-induced lid closing.

Catalytic Mechanism

Based on both the structural and biochemical observations, a detailed catalytic mechanism of ADK has been recently proposed (Park and Gupta 2008). The first step involves binding of inorganic phosphate or an activator compound to the conserved NXXE motif (Fig. 2.3a). The binding of an activator, in turn, facilitates binding of a free Mg^{2+} as well as the substrate adenosine to the active site of the “open” enzyme. The binding of adenosine turns on the GG switch, which results in the hinge-bending and the subsequent burial of the substrate. The closing of the lid then increases the enzyme affinity for MgATP, as seen with the translocation of Arg136 (Arg132 in human ADK) (Fig. 2.3b). MgATP binding induces formation of the anion hole, which stabilizes the pentacovalent transition state typical of an inline S_N2 displacement reaction. The catalytic magnesium ion enhances the electrophilicity of the γ -phosphorous in ATP by withdrawing charge via its interaction with the nonbridging oxygen atoms. The bound inorganic phosphate, on the other hand, may have a similar interaction with the β -phosphate of ATP. Due to the increased electronegativity of the γ - and β -phosphorous atoms, the electrons on the bridging oxygen are drawn away, making the covalent bonds weak and elongated. In the mean time, the catalytic base Asp318 (Asp300 in human ADK) deprotonates the 5'-hydroxyl end of adenosine, which subsequently attacks the positive center of the γ -phosphate. In the final step (Fig. 2.3c), the γ -phosphate is transferred to adenosine, and the products are released in the order of ADP and AMP as the adenosine-binding site is more deeply buried.

2.1.2.2 Inhibitors of Adenosine Kinase as Therapeutic Agents

The recent interest in ADK function and structure is largely due to its potential as a pharmacological target. As mentioned previously, adenosine shows a broad range of tissue-protective effects as a neuromodulator. In tissues under adverse conditions, the intracellular production of adenosine is dramatically increased (Williams and Jarvis 2000). Increased cytosolic concentration results in the net release of adenosine into the extracellular space via bidirectional ENTs (Griffith and Jarvis 1996). By activating various adenosine receptors on the cell surface, adenosine acts to limit tissue damaging and restore normal function (Ralevic and Burnstock 1998). Utilization of exogenous adenosine for pharmacological purposes, however, is difficult to achieve due to its short half-life in the interstitial fluid, which is on the order of seconds (Moser et al. 1989). Systematic introduction of adenosine receptor agonists have also proven limited, due to the detrimental side effects caused by the

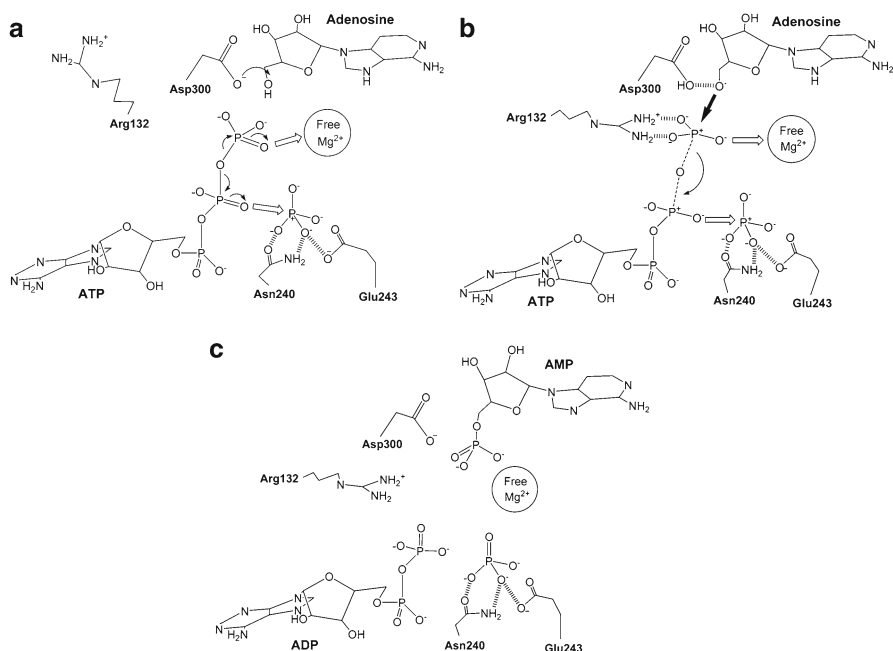


Fig. 2.3 Catalytic mechanism of adenosine kinase. Binding of inorganic phosphate facilitates binding of free Mg^{2+} as well as the substrate adenosine to the active site. This induces closing of the lid domain, which in turn increases the enzyme affinity for the second substrate ATP. The Mg^{2+} ion complexed with ATP is not shown in the scheme. *Hatched lines* indicate hydrogen-bonds. (a) Once ATP is bound, the phosphate molecule enhances the electrophilicity of the β -phosphorous atom by withdrawing charge via its interaction with the nonbridging oxygen of the β -phosphate. The catalytic magnesium ion, on the other hand, may have a similar interaction with the γ -phosphate of ATP. Due to the increased electronegativity of the β - and γ -phosphorous atoms, the electrons on the bridging oxygen are drawn toward the phosphorous atoms, making the bonds between the β - and γ -phosphate weak and elongated. In the mean time, the catalytic residue Asp300 deprotonates the 5'-hydroxyl of adenosine. (b) The positive center of the γ -phosphate attracts the negative charge developed on the 5'-hydroxyl of adenosine after proton abstraction by the catalytic base Asp300. Arg132 helps stabilize the negative charges on the nonbridging oxygen atoms of the γ -phosphate. (c) The γ -phosphate is transferred to adenosine. Arg132 flips back, and the products ADP and AMP are released

activation of adenosine receptors in nontarget tissues (Williams 1996). Inhibition of ADK, which can help maintain the elevated level of adenosine in the extracellular compartment (Pak et al. 1994), has been proposed as a promising alternative to exploit the adenosine receptor-mediated tissue protection. Since the release of endogenous adenosine is highly localized to the tissues under stress and trauma, the effects of ADK inhibition may only be pronounced at these specific sites (Kowaluk and Jarvis 2000). In view of this potential as a pharmacological agent, possibly without the adverse side effects associated with direct-acting adenosine receptor agonists, there has been a great interest and effort to identify and optimize ADK inhibitors.

Nucleoside Analogs

One of the most extensively characterized groups of ADK inhibitors consists of structural analogs of adenosine, such as 5'-amino,5'-deoxyadenosine (5'NH₂dAdo), 5-iodotubercidin (5IT) and 5'-deoxy,5-iodotubercidin (5'd-5IT). With IC₅₀ values on the order of several nanomoles, these compounds are highly potent in inhibiting ADK in vitro (Kowaluk and Jarvis 2000). Although these compounds have shown positive effects in several animal models of pain, seizure, and ischemia (Miller et al. 1996; Jiang et al. 1997; Kowaluk et al. 1999; Wiesner et al. 1999), their pharmacological utility is largely compromised due to short plasma half-lives, poor bioavailability, lack of target selectivity, and the potential to form cytotoxic metabolites (Cottam et al. 1993; Wiesner et al. 1999). The therapeutic limitations are due to the fact that these adenosine analogs can also function as the substrates of ADK and/or agonists of the adenosine receptors. Additionally, these compounds can also potentially inhibit the transport or movement of adenosine into or to the outside of the cells.

In an effort to optimize the aforementioned ADK inhibitors for better in vivo properties, a large series of 5'NH₂dAdo and 5IT derivatives were synthesized and tested (Ugarkar et al. 2000; Bookser et al. 2005). Rounds of structure–activity relationship studies identified a number of diaryltubercidin analogs as potent inhibitors of ADK. Compounds such as GP790 (Ugarkar et al. 2003) and GP3966 (Boyer et al. 2005), for example, showed inhibition of human recombinant ADK at low nanomolar concentrations. When tested for anticonvulsant, anti-inflammatory, and analgesic properties in animal models, these compounds showed high efficacy with relatively mild adverse effects, which included decreased locomotor activity and muscular flaccidity. The traditional ADK inhibitors and adenosine receptor agonists, on the contrary, generally exert profound hemodynamic effects at equieffective doses and have much lower LD₅₀ values (Malhotra and Gupta 1997; Wiesner et al. 1999). However, the clinical trial for GP3966 was stopped due to a preliminary report of CNS hemorrhage in rats and dogs (McGaraughty and Jarvis 2006). GP3269, a similar ADK inhibitor, on the other hand, has been studied in man (Phase I), but the results have not been disclosed (Kowaluk and Jarvis 2000). In spite of the promising preclinical data, the efficacy and safety of this class of compounds in clinical settings have yet to be verified.

Nonnucleoside Inhibitors

In addition to optimizing nucleoside analog ADK inhibitors, there has been a great effort in identifying nonnucleoside ADK inhibitors. The rationale for such an endeavor is that with compounds that are effectively different from adenosine, side effects due to the systemic activation of adenosine receptors would be minimal. Some of the most promising nonnucleoside ADK inhibitors belong to two different classes, the pyridopyrimidine and the alkynylpyrimidine derivatives. These two groups of compounds were initially derived from a single high-throughput screening lead (Jarvis et al. 2000), and have been subjected to rigorous rounds of structure–activity

relationship studies (Lee et al. 2001; Matulenko et al. 2007). Compound ABT-702, an example of pyridopyrimidine with the IC_{50} value of 1.7 nM (Jarvis et al. 2000), is as comparably efficient as morphine for pain (Jarvis et al. 2000; Kowaluk et al. 2000) and has shown to attenuate cardiac hypertrophy (Fassett et al. 2011) in animal models. An alkynylpyrimidine compound, 5-(4-(dimethylamino)phenyl)-6-(6-morpholin-4-ylpyridin-3-ylethynyl)pyrimidin-4-ylamine, which shows a similar level of efficacy as ABT-702, has been crystallized with human ADK (Muchmore et al. 2006). Some of these pyridopyrimidine and alkynylpyrimidine derivatives, in animal studies, showed little effects on motor coordination, exploratory locomotor activity, mean arterial pressure, and heart rate, at effective doses for pain relief, likely due to lack of nucleoside-like structural features (Kowaluk and Jarvis 2000; Zheng et al. 2003).

Additionally, a group of pyrrolbenzoxa(thia)zepinones has recently been identified as a new class of nonnucleoside ADK inhibitors (Butini et al. 2011). These compounds are novel in that they function allosterically (i.e., binding at a site away from the active site), in contrast to all of the aforementioned compounds which are competitive (with respect to adenosine) inhibitors. Therefore, they are not likely to act as adenosine receptor agonists. These compounds also showed no affinity for the enzyme ADA and a low cytotoxic potential on cultured murine cells. If proven safe, ADK inhibitors may represent a new group of multi-purpose drugs, with analgesic, anti-inflammatory, and antiepileptogenic properties, as well as cardioprotective effects.

We have also carried out limited work on screening of chemical libraries to identify novel nonnucleoside inhibitors of ADK. These studies have identified a number of compounds not belonging to the pyridopyrimidine or alkynylpyrimidine groups of compounds that inhibited ADK in submicromolar range. All four of these compounds (viz. 2-tert-Butyl-4*H*-benzo[1,2,4]thiadiazine-3-thione; *N*-(5,6-Diphenyl-furo[2,3-*d*]pyrimidin-4-yl)-propionamide; 3-[5,6-Bis-(4-methoxy-phenyl)-furo[2,3-*d*]pyrimidin-4-ylamino]-propan-1-ol; and 2-[2-(3,4-Dihydroxy-phenyl)-5-phenyl-1*H*-imidazol-4-yl]-fluoren-9-one) inhibited human ADK both in vitro and in cultured cells, without exhibiting any cellular toxicity in the effective dose range (Park et al. 2007).

2.1.2.3 Isoforms of Adenosine Kinase and Subcellular Localization

There are two isoforms of ADK in various mammalian organisms including humans (Juranka and Chan 1985; Szychala et al. 1996; Singh et al. 1996; McNally et al. 1997). The recombinant proteins of both forms are enzymatically functional and show no differences in their kinetic properties (Sakowicz et al. 2001; Sahin et al. 2004). The two isoforms are identical except at the N-terminus, where the long ADK isoform (ADK-L) contains extra 20–21 amino acids (MAAAEEEPKPKKLLK VEAPQAL in human ADK) that replace the first four amino acids (MTSV in human ADK) in the short isoform (ADK-S). Most programs for prediction of cellular localization (e.g., PSORT, BaCelLo) revealed no specific localization of these isoforms, and thus they were both assumed to be present in the cytoplasm (Nakai and Horton 1999; Sakowicz et al. 2001). Recently, it has been shown that ADK-L is localized

within the nucleus, whereas ADK-S is found in the cytoplasm (Cui et al. 2009). The extra 20–21 amino acids present at the N-terminus of ADK-L were capable of directing other proteins to the nucleus (Cui et al. 2009), thus qualifying as a new, nonclassical nuclear localization signal.

Tissue Distribution

The two ADK isoforms have shown to express differentially in mammalian tissues (Sakowicz et al. 2001; Sahin et al. 2004; Cui et al. 2011). In the most recent tissue-distribution study (Cui et al. 2011), both the long and short isoforms were shown to express in comparable amounts in rat liver, kidney, pancreas, and lung. The heart, spleen, skeletal muscle, and thymus, in contrast, showed expression of mainly the long isoform. Of the different tissues examined, the brain was the only tissue in which the short isoform was predominantly expressed. The overall level of ADK expression, on the other hand, has shown to be the highest in the liver and decrease in the kidney, lung, heart, brain, and skeletal muscle, in the order of appearance, in both rats and mice (Sakowicz et al. 2001; Sahin et al. 2004; Cui et al. 2011).

Involvement in Methylation Processes

The differential expression of the ADK isoforms and the observed differences in their subcellular localization suggest that they are involved in different physiological processes or functions. Interestingly, the enzyme ADK, in addition to its well-studied role in the purine salvage pathway, also plays an essential role in facilitating various methyltransferase reactions (Boison et al. 2002). All transmethylation reactions catalyzed by *S*-adenosylmethionine (SAM)-dependent methyltransferases result in the production of *S*-adenosylhomocysteine (SAH) (Fig. 2.1). SAH, which is also a powerful inhibitor (product inhibition) of the SAM-dependent methyltransferases, needs to be removed efficiently to ensure the continuation of transmethylation reactions. The enzyme responsible for the removal of SAH is SAH-hydrolase, which converts SAH to adenosine and homocysteine (Fig. 2.1). As SAH-hydrolase action is reversible and adenosine concentration-dependent, rapid removal of the inhibitory end product adenosine by ADK is essential for the SAM-dependent methylation reactions (Kredich and Martin 1977; Boison et al. 2002). The nuclear localization of ADK-L, therefore, suggests that this isoform is involved in DNA methylation and needs to be in close proximity with other methylation machineries (Cui et al. 2009). Other key enzymes involved in DNA methylation, such as DNA methyltransferase and SAH-hydrolase, are also localized in nucleus (Jue et al. 1995; Kloor et al. 2007). In contrast to ADK-L, the cytoplasmically localized ADK-S is likely involved mainly in purine salvage and keeping the adenine nucleotides pool in balance (Cui et al. 2009).

DNA methylation is one of the main epigenetic regulation mechanisms that operate throughout the development of organisms. In animals, it is most active at the

embryonic and postnatal stage, during which the promoters of specific genes are turned on/off at strict time points (Reik 2007). In the ADK knockout mouse, the fine tuned epigenetic regulatory process is likely disturbed due to the failure in adenosine removal, and this is one of the possible reasons for its neonatally lethal phenotype (Boison et al. 2002). The importance of the biochemical link between ADK and methylation has been well established in plants as well (Moffatt and Weretilnyk 2001). ADK knockdown (7–65 % of the WT ADK activity) in *Arabidopsis thaliana*, for example, produced dramatic and detrimental developmental abnormalities (Moffatt et al. 2002; Pereira et al. 2006). The essential role of ADK in the methylation reactions also provides a plausible explanation as to why, in contrast to other enzymes in the purine salvage pathway, such as ADA, purine nucleoside phosphorylase, and hypoxanthine-guanine phosphoribosyltransferase, whose deficiency leads to a variety of genetic disorders (Boss and Seegmiller 1982), no mutations or diseases linked to the ADK locus have been identified in humans.

2.1.2.4 Mutants Affected in Adenosine Kinase

Unlike in whole organisms, mutants affected in ADK can be readily obtained in cultured cells by selecting in the presence of toxic concentrations adenosine analogs (Gupta 1989). ADK mutants survive in such conditions, as they are unable to convert the analogs into the toxic, phosphorylated forms. Based upon their chemical structures, these adenosine analogs can be grouped into two broad classes. Compounds such as tubercidin and toyocamycin, like adenosine, contain a carbon–nitrogen bond in the ribosidic linkage and are referred to as N-nucleosides. Other analogs such as formycin A and formycin B, in which a carbon–carbon bond links the ribose sugar to the purine base, are referred to as C-nucleoside (Daves and Cheng 1976).

Mutants of mammalian cells have been selected from several cell types (Gupta and Siminovitch 1978; Gupta and Singh 1983; Mehta and Gupta 1983, 1985; Juranka and Chan 1985; Gupta and Mehta 1986; Cui et al. 2011). Most of these mutants contain no detectable ADK activity in the cell extract and exhibit high levels of cross-resistance to various N- and C-nucleosides. The resistance phenotype of these mutants (Class A) behaves recessively in the cell hybrids formed with sensitive cells, as the WT enzyme from the sensitive cells can phosphorylate and activate adenosine analogs. Another group of mutants (Class B) exhibit cross-resistance to various C-nucleosides while remaining sensitive to N-nucleosides. What is intriguing about the Class B mutants is that, although these cells are sensitive to N-nucleosides, no ADK activity (i.e., phosphorylation of adenosine, which is also an N-nucleoside) is detected in their cell extracts. Further, their drug-resistant phenotype behaves dominantly in the cell hybrids formed with either WT cells or Class A mutant cells, as both types of the hybrids are resistant to C-nucleosides but sensitive to N-nucleosides. Other mutants affected in ADK (Class C) show increased resistance to certain adenosine analogs, of which they exhibit reduced phosphorylation in the cell extracts.

Molecular Characterization of Mutants

Molecular characterization of various ADK mutants has been recently carried out. Many of the Class A mutants (e.g., Tub^R-1, Tub^R-3, Tub^R-5, Toy^R-3, and Toy^R-4, derived from Chinese hamster ovary (CHO) cells) have shown to contain large deletions in the ADK gene, which result in inactive, truncated proteins or no expression of the enzyme at all (Singh et al. 2001; Singh and Gupta 2004; Cui et al. 2011). Some Class A mutants, however, show normal expression of intact, but catalytically inactive ADK, which probably is affected by site-specific mutations. DrToy^R-18 (also derived from a CHO cell line), for example, contains a single base substitution (G to A) that changes Glu242 in the conserved NXXE motif to Lys (Cui et al. 2011). As discussed earlier, the NXXE motif is involved in the binding of the activating phosphate and magnesium ions, and the complete lack of activity in the mutant enzyme provides direct evidence for the importance of this motif.

Of the Class B mutants, the molecular alteration in Fom^R-4 (a CHO cell line derived mutant, selected against formycin A) has been characterized (Cui et al. 2011). This mutant contains in ADK a mutation of Ser191 to Phe, which is located near the adenosine-binding pocket. The C-nucleosides, to which the Fom^R-4 mutant exhibits selective resistance, exist predominantly in Syn conformation, while adenosine and other N-nucleosides do so in anticonformation (Daves and Cheng 1976; Gupta 1989). Hence, it is likely that this molecular alteration selectively prevents the binding of C-nucleosides to the mutant enzyme (Cui et al. 2011). This account, however, cannot explain the two puzzling, aforementioned aspects of the mutant: the lack of ADK activity in the cell extract and the dominance of its resistance phenotype in cell hybrids. Although the underlying biochemical and molecular mechanisms for these phenomena are not clear, it is possible that the mutant enzyme interacts and/or forms a complex with a downstream enzyme such as adenylate kinase (Cui et al. 2011). Adenylate kinase, which carries out the reaction $\text{AMP} + \text{ATP} \leftrightarrow 2 \text{ADP}$, and nucleoside diphosphate kinase, which catalyzes the subsequent phosphorylation of ADP to ATP, are important for the toxicity of adenosine analogs, as it is the di- and triphosphate forms of these compounds that get into and interfere with different metabolic pathways (Gupta 1989). In this context, it should be noted that the S191F mutation is present on the surface of the protein and is replacing a hydrophilic residue with a hydrophobic one. The surface hydrophobic patch created here may be responsible for the proposed protein–protein interaction (Cui et al. 2011).

Mutants VF18 and VF19 (derived from Chinese hamster V79 cells), which also exhibit preferential resistance to C-nucleosides, have been characterized as well (Cui et al. 2011). The molecular alterations observed in these mutants are L188F in VF18 ADK and L188F as well as F221L in VF19 ADK. As these residues are near the adenosine-binding site, it is likely that they also selectively prevent the binding of C-nucleosides to the mutant enzymes. The mutants VF18 and VF19 are also of much interest, because in contrast to all other ADK mutants, they are the only mutants exhibiting increased ADK activity relative to the parental V79 cells in cell extracts. Further, these mutants show higher expression of ADK-S in comparison

to ADK-L, whereas the parental V79 cells show higher expression of ADK-L. These observations indicate that VF18 and VF19, in addition to the molecular changes identified in the coding region, also contain additional genetic changes affecting the expression of the two isoforms. To fully understand the functional significance of different changes in the ADK mutants, further characterization of these mutants must be carried out at genetic, molecular, and biochemical levels.

2.1.2.5 Adenosine Kinase Gene Structure

The gene structure of ADK has been at least partially characterized in several organisms (Singh et al. 2001; Singh and Gupta 2004; Cui et al. 2011). In various mammalian species (viz. human, mouse, and Chinese hamster), the gene for ADK-L is linked in a head-to-head manner with the gene for the μ 3A subunit of the adaptor protein complex-3 (AP-3), and both these genes are transcribed from a single bidirectional promoter (Singh and Gupta 2004). The AP-3 μ 3A adaptor protein is involved in vesicle-mediated protein sorting, and an alteration in this protein has been observed in Hermansky–Pudlak syndrome, a genetic disorder characterized by defective lysosome-related organelles (Dell’Angelica et al. 2000a,b). The possible significance of the close linkage of these two genes and their sharing a common promoter is presently not clear. The promoter region as well as the first exon for ADK-S, on the other hand, lies within the first intron of the ADK-L gene (Cui et al. 2011). The ADK-L and ADK-S isoforms are identical except for the amino acids encoded by their first exons and are thought to arise by differential splicing of their unique first exons and the other common exons. The fact that each of these first exons has their own promoter strongly suggests that the expression of the two ADK isoforms is independently regulated at the transcriptional level. It is of much interest to further characterize the promoter regions as well as various transcriptional regulatory elements of the two isoforms to understand the specific factors or triggers that are responsible for differential expression of these two isoforms in different tissues under various conditions.

Another interesting aspect of ADK genes in various organisms is their extremely large size (Singh et al. 2001; Singh and Gupta 2004). The human ADK is around ~550 kb long, and the genes from rat, mouse, and Chinese hamster are also very large. Both human and Chinese hamster ADK genes have identical structures, consisting of 11 exons, whose lengths range from 36 to 173 bps and total to 1.1 kb. The lengths of the intervening introns in the human genome vary from 4.2 to 128.6 kb, averaging ~50 kb. The ratio of the noncoding to coding sequence for human ADK (i.e., >550) is the highest known for any gene in mammalian or other organisms. Recent analyses (unpublished results) of various animal genomes show that the large size of the ADK gene is a unique characteristic of the amniotes (i.e., tetrapod vertebrates that have a terrestrially adapted egg, including various mammals, birds, and reptiles). On the contrary, ADK genes in other eukaryotic organisms are small (Singh et al. 2001; Singh and Gupta 2004). ADK genes in amphibians and fish, for example, are ~20–25 kb. *Drosophila melanogaster* and *Caenorhabditis elegans*

ADK genes are only 1.5 and 1.3 kb long, containing two and four small introns, respectively. *A. thaliana*, on the other hand, has 10 small introns in its ADK gene, whose total length is 2.4 kb.

The large size of the ADK gene in humans and other amniotes raises many interesting questions about its biological significance. Based on its size, it is estimated that the transcription of the human ADK gene should take about 4 h (Tennyson et al. 1995). It is possible, therefore, that this gene serves as some kind of developmental timer or checkpoint to ensure the completion of some critical steps during development (e.g., transcription of certain genes that may be methylated subsequently). Further, although the human genome (NCBI) database currently indicates that no other genes are present within the 546 kb region covered by the ADK gene, a recent analysis (unpublished) has identified within its introns several large ORFs that exhibit high degrees of sequence similarity to known proteins. The cellular functions of these predicted proteins and their potential involvement in the regulation of ADK remain to be determined.

2.1.2.6 Adenosine Kinase and the Ribokinase Family Proteins

Sequence comparisons with other enzymes have shown that ADK belongs to the ribokinase family of carbohydrate kinases, which phosphorylates the hydroxymethyl groups of a variety of sugar moieties (Wu et al. 1991). The ribokinase family forms one of the three nonhomologous branches of sugar kinases, along with the hexokinase family and the galactokinase family (Bork et al. 1993). At the time of its identification, the ribokinase family consisted of only a small number of evolutionarily related enzymes. Besides ribokinase from *E. coli* and yeast, other members of this group included fructokinase, 1-phosphofructokinase, 6-phosphofructo-2-kinase (PfkB or PFK2), tagatose-6-phosphate kinase, and inosine kinase (INGK) (Bork et al. 1993). As more proteins were sequenced and characterized, a number of other enzymes were soon added to the ribokinase family, including ADK (Spychala et al. 1996; Singh et al. 1996) and 2-dehydro-3-deoxygluconokinase (Hugouvieux-Cotte-Pattat et al. 1994). It should be noted that nucleoside kinases, such as ADK and INGK, are also sugar kinases in that they phosphorylate the sugar moiety of the nucleosides. The proteins of the ribokinase family typically range from 280 to 430 amino acid residues in the total length (Park and Gupta 2008).

The members of the ribokinase family are now identified by signature sequences in the two highly conserved motifs (Fig. 2.4a) (Park and Gupta 2008). The first motif is found in a glycine-rich area located in the N-terminal region of these enzymes. Crystallographic data for ADK from various sources as well as other family members (Mathews et al. 1998; Sigrell et al. 1998; Schumacher et al. 2000; Arnfors et al. 2006; Cabrera et al. 2010), indicate that this motif includes two structurally significant consecutive glycine residues, the GG switch. As described earlier, this GG dipeptide is a part of the hinge between the $\alpha\beta\alpha$ domain and the lid domain, which undergoes torsional changes upon the sugar substrate binding. The second motif is found in the C-terminal region. Structural evidence suggests that these residues

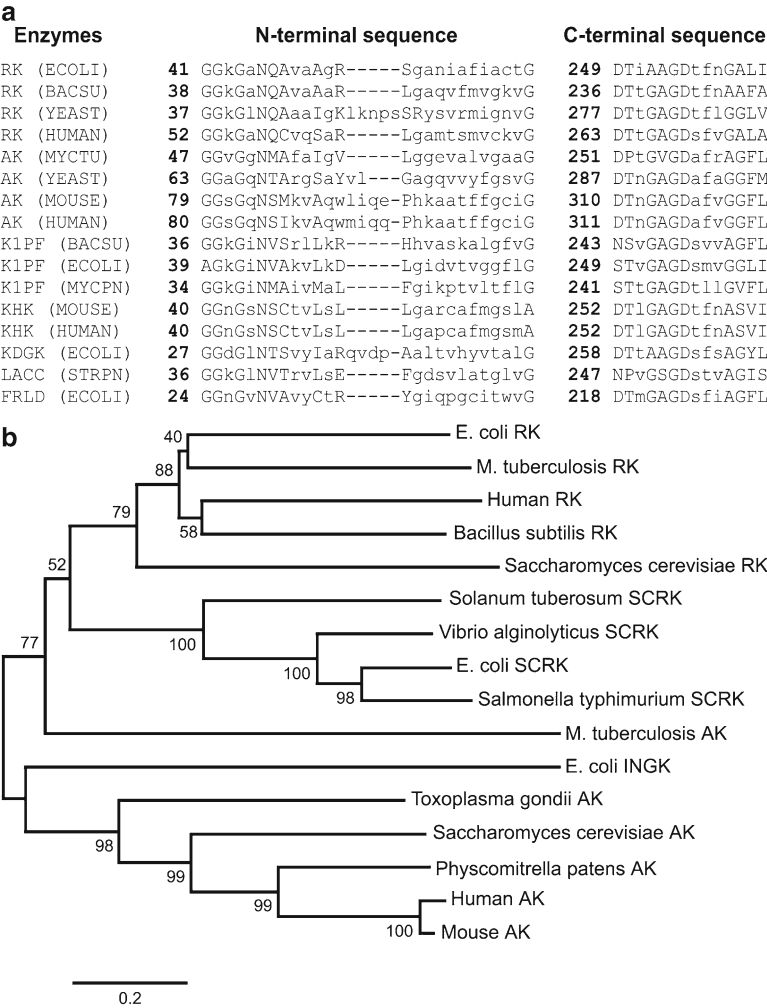


Fig. 2.4 The sequence motifs characteristic of the ribokinase family of proteins. **(a)** Conserved signature sequences of the ribokinase family enzymes. Conserved amino acid residues are shown capitalized in the alignment. Abbreviations for the enzymes: *RK* ribokinase, *ADK* adenosine kinase, *K1PF* 1-phosphofructokinase, *KHK* ketohexokinase, *KDGK* 2-dehydro-3-deoxygluconokinase, *LACC* tagatose-6-phosphate kinase, *FRLD* fructoselysine kinase. Abbreviations for the species: *ECOLI* *Escherichia coli*, *BACSU* *Bacillus subtilis*, *MYCTU* *Mycobacterium tuberculosis*, *MYCPN* *Mycoplasma pneumoniae*, *STRPN* *Streptococcus pneumoniae*. The amino acid numbering of mouse and human *ADK* is for the long isoform. **(b)** A consensus neighbor-joining tree for the ribokinase family of proteins. The numbers on the nodes are bootstrap scores. The abbreviations for the enzymes are: *RK* ribokinase, *SCRK* fructokinase, *ADK* adenosine kinase, *INGK* inosine-guanosine kinase

are involved in ATP binding and the formation of the anion hole. This sequence motif also contains an absolutely conserved aspartic acid residue which serves as the catalytic base (Asp317 in human ADK-L). Although the overall sequence identity between the family members is less than 30 %, the crystal structures of the ribokinase family members are extremely similar, retaining the overall structure composed of the large $\alpha\beta\alpha$ sandwich domain and the small lid domain (Park and Gupta 2008). Enzymes in this group differ only to a small extent structurally, in the sugar substrate binding moiety and minor peripheral structures. Advances in sequencing technology have led to a rapid increase in the number of enzymes that belong to the ribokinase family. New members include fructoselysine kinase, the bifunctional enzyme D-hepta-D-heptose-7-phosphate kinase/D-hepta-D-heptose-1-phosphate adenosyltransferase, and a number of uncharacterized sugar kinases mostly of bacterial sources (Park and Gupta 2008).

Interestingly, the ability to utilize adenosine as a substrate appears to have arisen at least twice in the ribokinase family. ADK activity until recently was only found in the eukaryotic organisms. However, a recently identified enzyme from *Mycobacterium tuberculosis* has been denoted as the first, and thus far the only, bacterial ADK (Long et al. 2003). Sequence alignment identified some regions in this *M. tuberculosis* enzyme that are conserved in ADK from other sources. Analysis of the aligned sequences suggests that the conserved residues are involved in the binding of the substrates adenosine and ATP (Long et al. 2003). The *M. tuberculosis* enzyme, however, shows greater overall sequence homology with ribokinase and fructokinase (35 %) than it does with other ADKs (less than 24 %). Other ADKs, in contrast, show more than 50 % sequence homology with one another. In a phylogenetic tree of the ribokinase family, *M. tuberculosis* ADK branches with ribokinases and fructokinases, while ADKs from other sources form a separate branch (Fig. 2.4b) (Park et al. 2009). The substrate specificity of this *M. tuberculosis* enzyme is also very different from that of other eukaryotic ADKs (Park et al. 2009). For example, while human ADK favors adenosine approximately 900-fold over ribose as a substrate, *M. tuberculosis* ADK does so only about ninefold. Further, the *M. tuberculosis* enzyme can also phosphorylate fructose as effectively as it can phosphorylate ribose. Based on the results of the sequence alignment and biochemical studies, as well as the fact that ADK is not found in other bacteria, the ADK activity exhibited by the *M. tuberculosis* enzyme appears to have resulted from convergent evolution.

2.1.3 Concluding Remarks

The enzyme adenosine kinase plays a central role in the metabolism of adenosine and in determining its intra- as well as extracellular levels. In the past few decades, although much has been learnt about the structure of ADK, its catalytic mechanism and some aspects of its genomic organization, our understanding of the cellular functions of the two ADK isoforms and how their relative levels in various tissues that exhibit large differences are regulated is entirely lacking. Due to the proven

neuroprotective and cardioprotective effects of increased concentration of adenosine, inhibition of ADK is now widely recognized as an important strategy to benefit from this body's endogenous defense mechanism (Wiesner et al. 1999; Kowaluk and Jarvis 2000; Boison 2009a, 2011; Theofilas et al. 2011). However, due to the important role that ADK and adenosine play in different tissues and regulatory pathways, the compounds that are general inhibitors of ADK have proven of limited therapeutic value due to their undesired toxic effects. To develop more specific inhibitors, whose effects are limited to a desired function(s) of the ADK at a specific location (viz. tissue), it is important to understand the cellular functions of the two isoforms and different protein factors that are involved in their tissue-specific expression. The knowledge gained by these studies should prove valuable in designing specific means (inhibitors) to modulate the activity of this enzyme in a highly specific manner to realize the therapeutic potential of increased local concentration of adenosine for treatment of neuronal (viz. epilepsy, schizophrenia) as well as other diseases (Boison 2011; Boison et al. 2011). Another very interesting aspect of ADK that needs to be investigated relates to the unusually large size of its gene in the amniotes and the possible functions of several predicted proteins that are present within its large introns. These observations suggest that ADK might be playing an important role during development that is presently not understood. Lastly, despite the important roles of ADK in a number of different pathways, no genetic condition has been attributed to mutations of ADK thus far. The emerging role of ADK in the development of a number of neurological disorders (Boison 2010; Shen et al. 2010; Boison et al. 2011) suggests that it would be of interest to examine them for possible alteration in the ADK gene.

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