REVIEW

Dynamics of nucleotide metabolism as a supporter of life phenomena

Takafumi Noma

Department of Molecular Biology, Institute of Health Biosciences, The University of Tokushima Graduate School, Tokushima, Japan

Abstract : Adenylate kinase (hereinafter referred to as AK) catalyzes a reversible high-energy phosphoryl transfer reaction between adenine nucleotides. The enzyme contributes to the homeostasis of cellular adenine nucleotide composition in addition to the nucleotide biosynthesis. So far, six AK isozymes, AK1, AK2, AK3, AK4, AK5, and AK6, were identified. AK1 is localized in neuronal processes, sperm tail and on the cytoskeleton in cardiac cells at high concentrations, suggesting its regulatory function as a high-energy β-phosphoryl transfer chain from ATP-synthesizing sites to the ATP-utilizing sites in the cell. AK2, AK3 and AK4 are mitochondrial proteins. AK2 is expressed in the intermembrane space, while AK3 and AK4 are localized in the mitochondrial matrix. AK3 is expressed in all tissues except for red blood cells indicating that AK3 gene is a housekeeping-type gene. On the other hand, AK4 is tissue-specifically expressed mainly in kidney, brain, heart, and liver although its enzymatic activity is not yet detected. AK5 is solely expressed in a limited area of brain. AK6 is recently identified in nucleus, suggesting its role in nuclear nucleotide metabolism. All data, so far reported, indicated the function of AK is associated with the mechanism of efficient transfer of high-energy phosphate in micro-compartment within the cell. J. Med. Invest. 52 : 127-136, August, 2005

Keywords **:** *adenine nucleotide, adenylate kinase, high-energy phosphoryl transfer, homeostasis, isozyme*

INTRODUCTION

Nucleosides are formed by glycoside bonds between nitrogen bases such as purine and pyrimidine and reduced groups of sugars, while nucleotides are compounds formed by ester bonds between sugars of these nucleosides and phosphates. Nucleotides have two types of sugar residue, D-ribose and D-2-deoxyribose. In addition, basic residues include purine nucleotides (adenine and guanine) with purine bases, and pyrimidine nucleotides (thymine, cytosine, and uridine) with pyrimidine bases. As is widely known, ribonucleotides and deoxyribonucleotides are constituents of RNA and DNA, respectively. The important physiological function to remember is that these nucleotides also work as donors of phosphate groups in the living body. In particular, ATP, ADP, and AMP of adenine nucleotides are important substances for energy metabolism. In this article, I present recently discovered dynamic aspects of nucleotide metabolism involved in two major biochemical properties, energy metabolism and self-duplication, from the viewpoint of adenylate kinase, an enzyme involved in adenine nucleotide metabolism.

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Address correspondence and reprint requests to Takafumi Noma, M.D., Ph.D., Department of Molecular Biology, Institute of Health Biosciences, The University of Tokushima Graduate School, Kuramoto-cho, Tokushima 770-8504, Japan and Fax : +81-88-633-7326.

ENERGY METABOLISM

Living bodies carry out vital activities using chemical energy. The chemical energy is mainly obtained by generating ATPs, high-energy compounds, through

metabolic processes such as the glycolytic cycle, citric acid cycle, and oxidative phosphorylation, which are oxidative processes of reductive substances like dietary sugars. Energy transfer of these ATPs enables living bodies to carry out vital activities, including muscle contraction, neuronal excitation-conduction, active transport, and cell proliferation. Used ATPs change into ADPs, and are again converted to ATP to be recycled. In other words, coupling reactions using ATPs as intermediates are the center of the energy flow in the living body, and function to maintain vital activities.

ATPs are molecules that store energy in a readily available form, and with other adenine nucleotides, ADP and AMP, their concentrations are maintained constant in a normal state, while involved in cellular metabolic turnover. As stated in biochemical textbooks, concentration in cells of adenine nucleotides, ATP, ADP, and AMP are maintained at approximately 5 mM, 1 mM, and 0.1 mM, respectively (Table 1)(1).

Table 1. Intracellular nucleotide concentration

	mM		
	ATP	ADP	AMP
Rat hepatocyte	3.38	1.32	0.29
Rat skeletal muscle	8.05	0.93	0.04
Human erythrocyte	2.25	0.25	0.02
Rat neuron	2.59	0.73	0.06
E.coli	7.90	1.04	0.82

This suggests that a mechanism to maintain constant concentration, namely, a homeostatic mechanism, is working in cells. Adenylate kinase is the enzyme working to this end. This AK has the ability to generate 2ADP molecules from ATP and AMP or convert 2ADP molecules into ATP and AMP in a reversible reaction (ATP+ AMP↔2ADP) by transferring γ-phosphate of ATP to AMP (2-10). It is becoming clear from past studies that AK exists in organisms from bacteria to humans (2-10). Reportedly, prokaryotic cells such as bacteria, and eukaryotic yeasts have only one type of AK enzyme, and cannot survive without its activity, indicating the importance of AK in energy metabolism (11, 12). Meanwhile, multicellular organisms such as humans have acquired multiple isozymes of AK in the evolutionary process. When we started this study, the number of isozymes was 3, but lately it increased by 3 in the situation preceded by gene isolation, and the existence of six kinds of isozymes has been demonstrated today (Table 2).

AK1 exists in cytoplasm, and AK2 partly in cytoplasm but mainly in mitochondrial intermembrane space, and they both catalyze generation of 2 ADP molecules from ATP and AMP, while AK3 catalyzes 1 GDP or ADP molecule formation or the reverse reaction using GTP, not ATP, as a substrate for phosphate donation. This AK3 generates GDP and ADP using GTP produced by phosphorylation in the citric acid cycle at substrate level and AMP that exists in the matrix, respectively, and the generated GDP is utilized in the next cycle of the citric acid cycle, while the ADP is utilized as a substrate of mitochondrial ATP synthetase. Three groups, reported isozymes AK4, AK5, and AK6 one after the other. It was demonstrated that, among them, AK5 was expressed only in the brain, and existed in cytoplasm with the same activity as AK1, while AK4 existed in the mitochondrial matrix. In addition, more recently, AK6 was revealed to exist in the cell nucleus, and have similar characteristics to AK5 (13-16).

Table 2. AK isozymes

Isozyme	M.W	Localization	Tissue distribution
AK 1	22 K	cytosol	muscle, brain, heart, testis
AK 2	30 K	mitochondrial intermembrane space	liver, kidney, heart
AK 3	28 K	mitochondrial matrix	all tissues except for red blood cell
AK 4	29 K	mitochondrial matrix	kidney, liver, heart, brain
AK 5	22 K	cytosol	brain
AK 6	20 K	nucleus	adrenal gland

Figure 1 shows interactions of the AK isozyme system in energy metabolism, summarizing localization and functions. ATPs produced in the citric acid cycle and oxidative phosphorylation in mitochondria, or by the glycolytic cycle in cytoplasm are used after delivery to ATP consumption sites by simple diffusion or high-energy phosphate transfer by reciprocal conversion of adenine nucleotide by AK enzyme.

In the course of the research, the following 3 basic questions concerning AK were raised: 1) Dose AK form a functional complex or not ? 2) What's the significance of tissue or intracellular distribution in energy metabolism ? 3) What's the physiological significance of the whole system of AK isozymes ? Next, I introduce the latest research results.

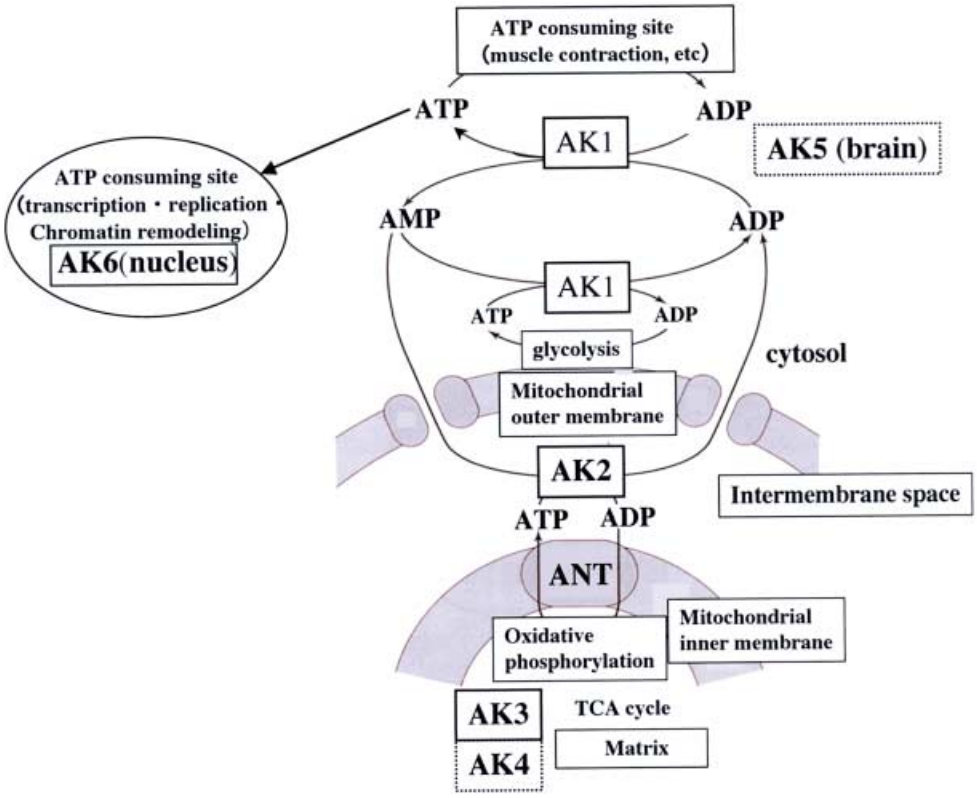


Figure 1 Energy metabolism and mutual relations of AK isozymes

EXPRESSION OF AK ISOZYMES

Northern blot analysis was conducted to examine the mRNA expression in mouse tissues, and revealed ubiquitous and constitutive expression of AK3, and in contrast, tissue specific expression of other AKs (Figure 2). AK1 is expressed especially in more energy-consuming organs, including the skeletal muscle, heart, testis, and brain, while high expression of AK2 and AK4 are confirmed in organs such as the liver and

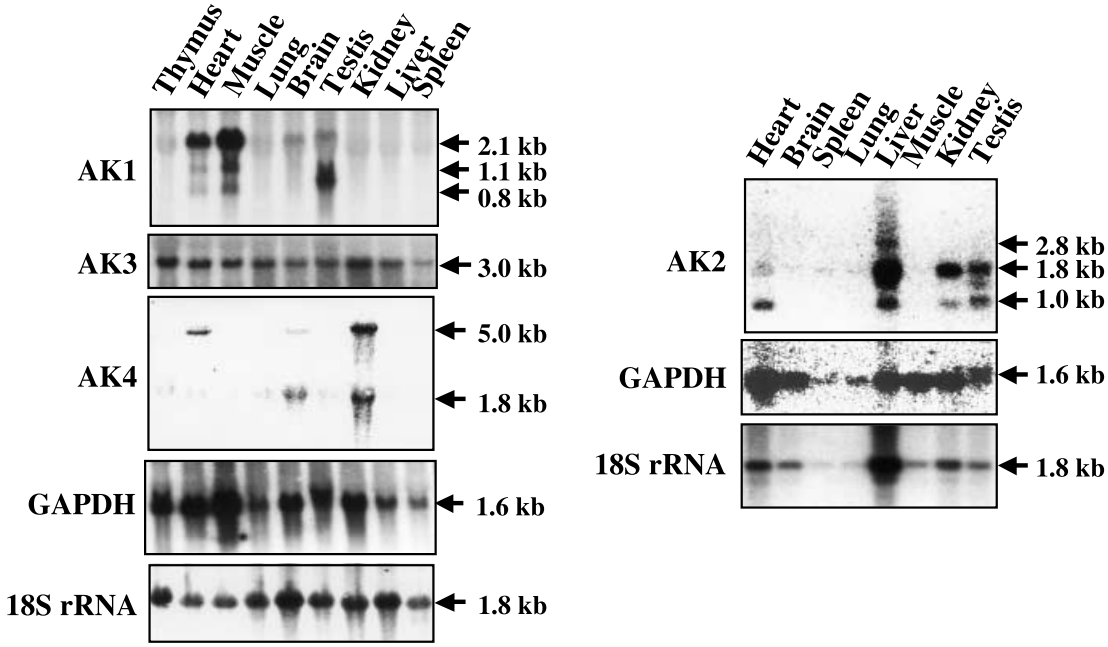


Figure 2 mRNA expression of AK isozymes
Northern blot was performed with each AK isozyme cDNA as a probe.

kidneys. Measurement of enzyme activities for each tissue revealed high correlation between enzyme activity of AK1 and its mRNA level, while activities of AK2 and AK3 didn't necessarily correlate with their mRNA quantities, suggesting post-transcriptional regulation (Figure 3)(10).

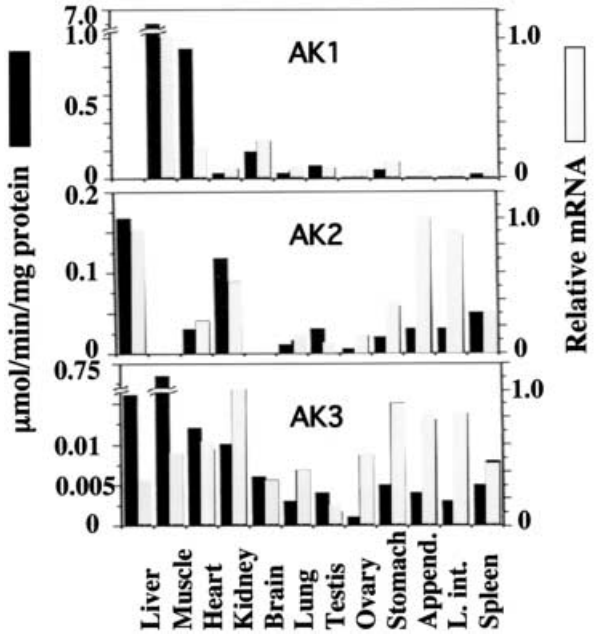


Figure 3 AK isozymes in rat tissues
Relative abundance of AK mRNA and enzyme activities are shown. The levels of AK activities are expressed as μmol/ min/mg protein of tissue homogenates. Relative abundance of AK mRNA are shown as blue bars. The letters" Append. "and" L.int. "indicate appendix and large intestine, respectively.

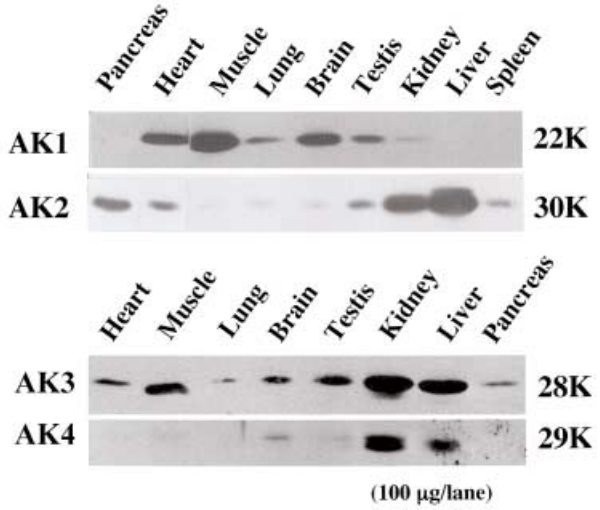


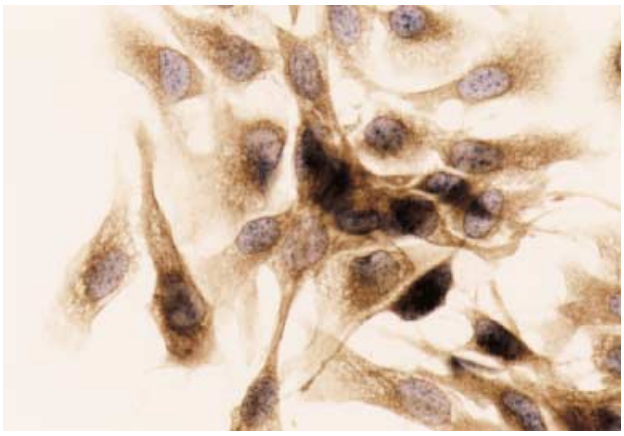
Figure 4 Tissue distribution of AK isozymes
Immunoblot analysis was performed to examine the expression of each AK isozyme in several tissues.

Expression of isozyme proteins in each tissue was examined by the immunoblot method, and as shown in Figure 4. AK1 is high in organs with high energy metabolism, including the skeletal muscle, brain, heart, and testis, AK 2 in the liver, kidneys, pancreas, and heart, AK3 in the liver, kidneys, and heart, and AK4 in the kidneys and liver, and in addition, near correlation between protein expression and mRNA levels was revealed.

Inouye *et al*. examined AK1-3 protein expression at each developmental stage in the brain, which has high energy metabolism (17). AK1 expression gradually increased just before birth, while AK2 expression was highest at fetal day 14, at which cells proliferate most rapidly, and decreased at birth to be undetectable. Meanwhile, gradual increase of AK3 expression from a low level was demonstrated (17). Therefore, a dominant role of AK1 isozyme was suggested in adenine nucleotide homeostasis in cytoplasm of neurons, in which energy metabolism is high. In addition, to examine the localization of AK1 in neurons, a primary culture of rat brain tissue was prepared, followed by reaction with anti-AK1 and anti-neurofilament antibodies, and staind. AK1 and neurofilaments were stained along neurites, demonstrating their concordant localization (18). Similar results were obtained using PC 12 cell, another model cell for neural differentiation, therefore, AK1 was considered to be expressed in concurrence with structural differentiation of neurons, and contribute to the functional maturation of neurons (19, 20).

For another tissue with active energy metabolism, cells derived from heart muscle were stained with AK1, and the staining pattern was observed, and seemed to coincide with the distribution of the cytoskeleton (Figure 5). In addition, staining properties of the testis and epididymis were examined (Figure 6), and AK2, 3, and 4 stained highly proliferating globular undifferentiated spermatocytes without tails, while AK1 intensely stained tails of differentiated sperms, which underwent complex structural changes. Besides, intense staining was observed in tail portion of sperms existing in the epididymis, revealing the coexistence of AK1 with the cytoskeleton that constitute the sperm tail, another high energy demanding cellular structure.

In summary, the above expression patterns demonstrated that 1) AK1 localizes in more energy-consuming organs and cells in concurrence with their differentiation, while AK2 localizes in rapidly proliferating cells, and 2) AK1 colocalizes with cytoskeletons. In other words, obtained results demonstrated colocalization of AK1 with cytoskeletons at high density, not even distribution in cells. Therefore, enzyme chain reactions



AK1

Figure 5 AK1 expression in cardiac derived cells
Immunocytochemical analysis of AK1 expression in H9c2, cardiac derived cell line indicates co-localization of AK1 and cytoskelton.

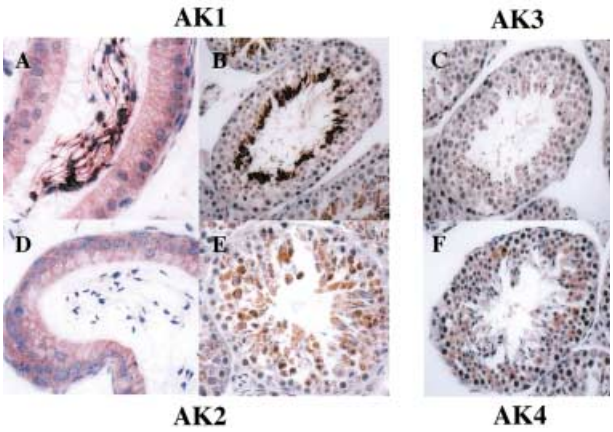


Figure 6 Expression of AK isozymes in testis and epididymis
AK 1 (A, B), AK 2 (D, E), AK 3 (C), and AK 4 (F) expression were examined. A and D in dicate epididymis. B, C, E, and F indicate testis.

cause reciprocal conversions of adenine nucleotides in local microenvironments, functioning to maintain the concentration balance among ATP, ADP, and AMP.

These results led to the invention of the high-energy phosphate transfer by AK model (Figure 7). It is known that cytoskeletons bind to organelles such as mitochondria, enzymes in the glycolytic cycle, and membrane bound proteins. This model depends on the idea that AK1 molecules binding to these cytoskeletons in some way receive ATPs from mitochondria, followed by transfer of reaction products to lower level ATP by a chain of reciprocal conversion reactions, and send ATP to ATPase that exists on the other ends of cytoskeletons. Also from the reports suggesting that cytoplasmic structure has completely different conditions from a solution within a glass, and is so complicated with macromolecules, including organelles and proteins, that movement of small molecules by simple diffusion is extremely slow (21), it is presumed that high-energy phosphate transfer is the effective mechanism to meet

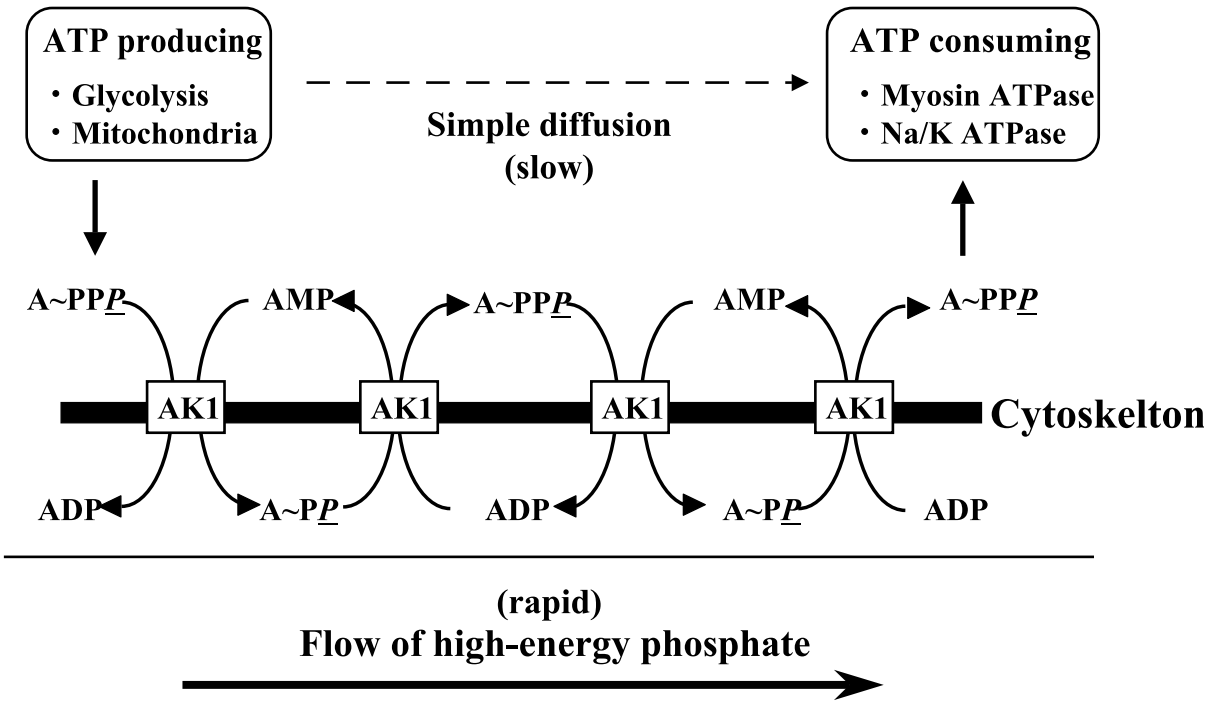


Figure 7 Model for high-energy phosphoryl transfer via AK chain reaction
A~PP*P* and A~P*P* indicate ATP and ADP, respectively. The underlined and italic phosphate indicate γ- and β-position, respectively. The model shows an efficient high-energy β-phosphoryl transfer from ATP producing sites to ATP consuming sites by AK-catalyzed chain reaction.

energy demands that change rapidly or dynamically at all times.

FUNCTIONAL ANALYSIS OF AK2 USING MODEL ORGANISM

Because of the many genetic analyses conducted on *Drosophila melanogaster*, which has nearly the same number of genes as the human genome, with many mutant strains maintained, relatively easy behavioral analyses and other reasons, *D. melanogaster* is often used as a model organism to unravel human disease mechanisms and to investigate functions of human body components such as organs and cells. In the life cycle of flies, the insects hatch at 24 hours after fertilization, become larva to grow through three larva stages, the first, second, and third instar, mainly by cell enlargement, and become a pupa on day 5 for 4 days, followed by ecdysis to be adult insects. It is therefore easy to detect abnormalities in differentiation and development, including behavioral analysis.

Northern blot analysis to examine the mRNA expression in the developmental process demonstrated a tendency for low *AK1* and *AK3* expression in embryos, increasing along with developmental progress, and slightly high *AK2* expression in embryos, with reduction in larva, increasing again in adults. Nearly consistent results of protein expressions were obtained by immunoblot analysis. In addition, it was demonstrated that AK1 protein expression in adult insects was higher in the chest and the head, which contain muscles and the brain, AK2 was high in the head, and AK 3 was expressed in all parts at the same level. AK2 expression is extremely low in the brain of mammals, while high in the head, which contains the brain in *D. melanogaster*, suggesting species-specific gene expression (22).

Therefore, to unravel the role of *AK2*, phenotypic analysis was conducted by *AK2*-mutants containing P-element insertion in the *AK2* gene region, resulting in unreadable *AK2* gene knockout. Insects that have this gene were not viable. To determine the time of death, and the survived number in the developmental process examined. Precise examination of the first, and second instar revealed that the fatality of insects lacking *AK2* gene was demonstrated between larva stages, the first, second, and third instar. Morphologically, larvae heterogeneous in *AK2* gene (*AK2*+/-) were far bigger than *AK2* gene null insects (*AK2*-/-) (Figure 8), almost the same size as wild type insects. This demonstrated significant growth suppression caused by lack of *AK2* gene.

Changes of enzyme activity in the developmental process showed that AK1 activity increased in the course of development, while that of AK2 and AK3 showed patterns of temporary decrease between embryo and larva stages, followed by increase. In addition, weak activity, approximately 1/3 activity of heterogeneous insects, was detected in null insects at the larva stages, first, and second instar (Figure 9).

Figure 8 Morphological appearance of mutant larvae
Three days later after fertilization, larvae were examined and separated. The left indicate the third instar larvae, which are *AK2* hetero-status. The right indicate the first and second instar larvae, which are *AK2* null-status.

Proteins were extracted from insects at each developmental stage, and AK-protein expression was examined by immunoblot analysis after electrophoresis

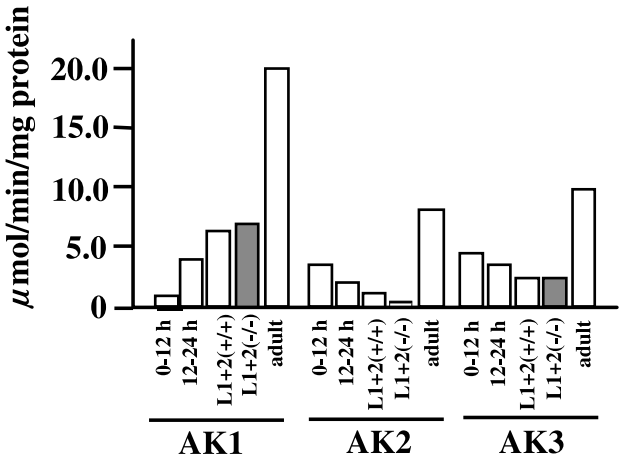


Figure 9 AK activity during *Drosophila* development
AK enzymatic activities were analyzed in protein extracts from embryo, larvae, and adult flies.

to confirm the existence of AK2 protein, leading to the presumption that AK 2 protein that existed in a fertilized egg as a maternal factor remained even at this stage, and disappearance of AK2 maternal factor stopped growth until death.

Next, whether or not behavioral changes might be caused by changes of *AK2* gene dosage was examined in association with energy metabolism, since heterogeneous insects with 1/2 gene dosage of *AK2* could grow to be adult insects. Usually, as shown in the pattern of the wild type, activity of flies reaches peaks at daybreak and dust, and each mutant fly also exhibited similar activities in bright and dark conditions by day 7. In constant dark condition, activity pattern of the wild type varied, while haploid exhibited a short cycle, shift of activity time earlier in the day. Meanwhile, a tendency of shift to a long cycle, latening of the activity time in the day, was observed in triploid flies (Figure 10)(22).

The results using the above model organisms suggested that AK2 was essential for survival and had effects on rhythm formation. Although how behavioral changes were associated with AK2 functions in the constant dark condition is unknown at the moment, the above results indicated the possibility that change in metabolic demand and supply of ATP was linked with rhythm formation, and AK2 functioned as a metabolic clock, since AK2 acts as a supplier of ADP, a substrate in ATP synthesis, to the mitochondrial matrix.

VARIOUS AK FUNCTIONS

1. Cystic fibrosis (CF) and AK

CF is an autosomal recessive inherited disease caused by a gene defect of cystic fibrosis transmembrane conductance regulator (CFTR), Cl- channel, and exhibits symptoms including refractory respiratory tract infection, pancreatic exocrine deficiency, chronic pancreatitis, and congenital male sterility, unfavorable symptoms with average life expectancy less than 30 years.

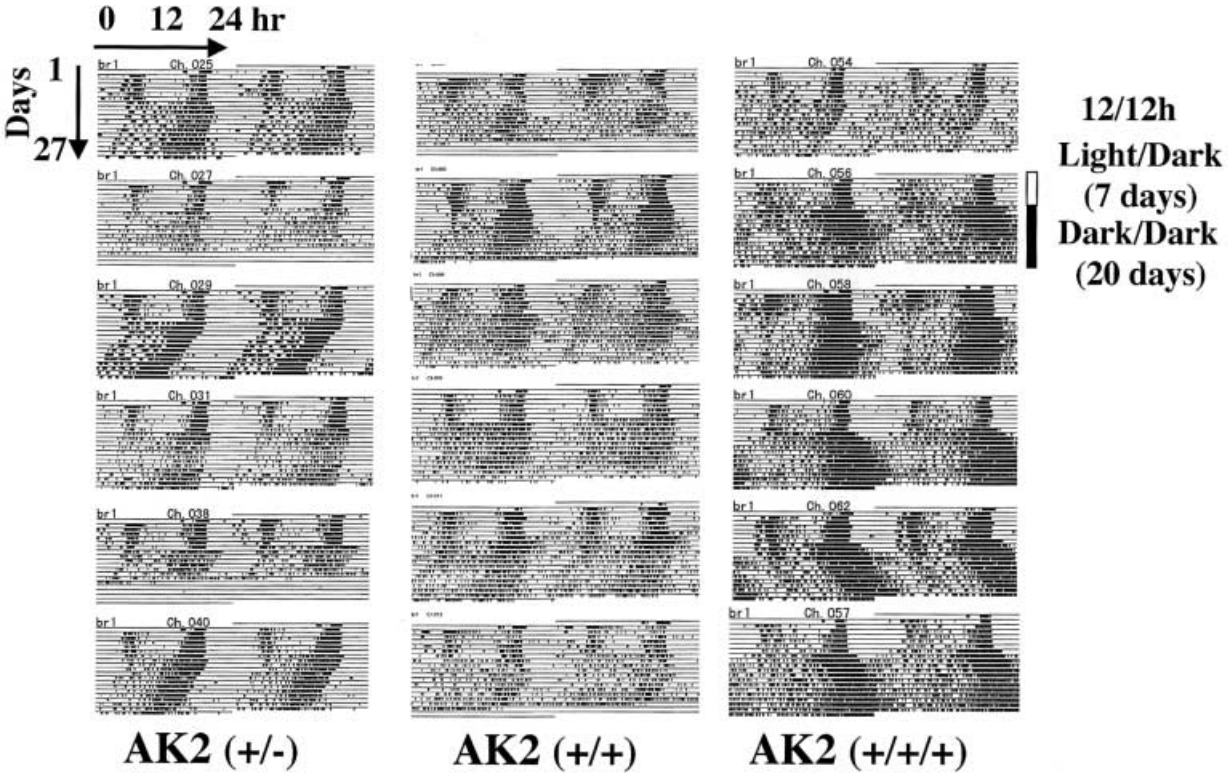


Figure 10 Locomotive activity of mutant fly
A horizontal arrow indicates 24 hours from 0:00 a.m. to 24:00 pm and a vertical arrow shows the days. The chart indicates a locomotive analysis for 27 days in duplicate. Three kinds of 30 flies, a hetero-individual, wild type, and a triplet-individual in *AK2* gene status, were analyzed. Here, the results of 6 examples are shown. Locomotive activity was measured under a cycle of 12 hour-dark and 12 hour-bright environment for first seven days. Thereafter, the activity was measured under 24 hour-dark environment from the eighth day. Locomotive activity is shown as a black vertical bar in one day column when a fly steps over one time of sensor.

The incidence of CF is about one in 350,000 in Japanese population (23), on the other hand, one in 2,500 among Caucasians (24). The Cl^- channel is a member of the ABC transporter family including the drug resistance gene MDR, and AK activity is confirmed at the intracellular domain of its carboxyl terminal, and it is becoming clear that the AK activity is involved in gating regulation for Cl^- ion passage (25).

Activities of the ABC transporter family, including multidrug resistance transporter gene products (MDR), are controlled by ATP and ADP adenine nucleotides, therefore, studies on activity regulation of ABC transporters by dynamic changes of nucleotides, including the presence or absence of AK activity, has attracted attention.

2. AK as an Ecto-enzyme in extracellular ATP metabolism

Clearance in the respiratory airway is carried out by stimulatory effects of airway fluid secretion and enhancement of ciliary movements. ATPs released from tracheal epithelial cells by mechanical stimuli delay the signals of ATPs to P2 receptors by slowing the degradation of ATP to adenosine, a reaction ATP+ AMP↔2ADP, by Ecto-AK enzymes released from cells. As a result, it is becoming clear that stimulatory effects of airway fluid secretion and enhancement of ciliary movements are prolonged by signals via the purinergic receptor, leading to the facilitation of clearance (26). Therefore, as a treatment of chronic bronchitis, AK is expected to be a molecule that leads to development of a new treatment through physiological adenine nucleotide metabolism, different to conventional treatments such as administration of antibiotics or expectorants.

In addition, functions of ATP metabolites as signaling molecules via AK give an impression that AK functions as an Ecto-enzyme in signaling via ATP receptors at neuronal synapses and neuronal junctions, and signaling via adenosine receptors in myocardial ischemia, thereby attracting attention.

3. Activity of chromatin structural conversion factor complex and AK

Chromatin structural conversion factor complex involved in tissue specific gene activation and suppression of cells has an ATPase domain homologous to the DNA and RNA helicase family. Changes of chromatin structure, including ATP dependent DNA transfer, require timely supply of ATP in the nucleus. Although the significance of the existence of nuclear AK6 has not been fully demonstrated, probably AK6 regulates changes of adenine nucleotide metabolism in the nucleus by maintaining homeostasis of nuclear adenine nucleotides, and therefore functions of this enzyme in the nucleus are attracting attention.

4. Nucleotides, nucleosides, and AK as an anticancer drug or antiviral drug

Nucleotides and nucleoside derivatives are attracting attention from the viewpoint of drug discovery. These molecules have already been on the market as anticancer or antiviral drugs. For example, it has been demonstrated that for 2-chloro-2'-deoxyadenosine (CdA) against hairy cell leukemia and 1-β-D-arabinofuranosylcytosine (AraC) against hematologic malignancy, AK and UMP-CMP kinase function as phosphorylation enzymes to activate each drug, and for AIDS drugs such as 2', 3'-dideoxyinosine (ddI) and 3'-azido-2', 3'-dideoxythymidine (AZT), AK, thymidine kinase, or deoxyTMP kinase activates each drug (27). Activation of nucleotides or nucleosides via phosphorylation results in inhibition of viral replication in infected cells, and growth inhibition or induction of apoptosis of tumor cells. Enzymes, which phosphorylate nucleotides or nucleosides, as well as AK, localize specifically in cell compartments, including cytoplasm, mitochondria, and nucleus, and it is useful to be able to utilize this localization for drug targets.

CONCLUSIONS

While many people have an illusion that" energy metabolism ", one of the main themes in biochemistry, has been resolved, it is intriguing to find, through investigation of functions of adenylate kinase, an old enzyme, that local energy transfer in cells might be generated by chain reactions of AK enzyme, and changes of energy metabolism might be linked with circadian rhythm, a theme of a seemingly very different area. In addition, the main research themes in life phenomena today, including signal transduction, expression regulation of genetic information, and regulation of cell proliferation and apoptosis, are linked as an extension of nucleotide and nucleoside metabolism, and therefore progress of this study in the future is expected.

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