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Adenylate Kinase:An Old Enzyme with New Aspects

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Abstract Adenylate kinase (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. We characterized cDNAs and determined the gene structures of three AK isozymes, AK1, AK2 and AK3. The genetic basis of a case of AK1 deficiency was clarified as a single-base substitution in one of the patient's chromosomes leading to the Arg to Trp change in the protein. We found that AK1 is localized in neuronal processes at high concentrations, which is probably related to a new regulatory function of AK as the high-erergy β-phosphoryl transfer chain from the ATP-synthesizing sites to the ATP-utilizing sites in the cell. AK2 and AK3 are mitochondrial proteins; the former is localized in the intermembrane space, while the latter is present in the matrix. We analyzed the transport mechanism of these AK isozymes into mitochondrial compartments and considered the physiological significance of the isozymes. Molecular evolution of the AK protein family is also discussed.

Prologue

When I was a graduate student in Professor Osamu Hayaishi's laboratory of Kyoto University, Jacques Monod's paper on 'allosteric enzymes' appeared in Journal of Molecular Biology in 1963.[1] At that time I was engaged in the study on threonine deaminase that was activated by adenine nucleotides. The deaminase isolated from an anaerobic bacterium, *Clostridium tetanomorphum* or of anaerobically grown *Escherichia coli* was activated by ADP or AMP, which was regarded as examples of allosteric activation.[2,3] The enzyme activation of this type was nicely interpreted by the energy charge model of the adenine nucleotide pool that was proposed by Daniel Atkinson.[4] Fig. 1 is a picture taken at the US-Japan Cooperative Seminar on Metabolic Control that was held in Kyoto in 1966. Professors Hayaishi and Atkinson were the organizers of this meeting to which Dr. Monod was invited from Paris. Since then I have constantly been interested in the adenine nucleotide homeostasis in living cells. Adenylate kinase (AK) plays a key role in the energy charge. In 1983, I started to analyze this enzyme with my colleagues of Yamaguchi University School of Medicine in Ube to understand the physiological significance of this enzyme. We were able to deepen understanding of the genetic back ground of AK. In this review I would like to

Table 1 AK isozymes in vertebrates

Isozyme	Molecular Weight	Subsellular Location	Tissue Distribution
AK1	22K	Cytosol	Muscle, Brain, Heart, Erythrocytes
AK2	28K	Mitochondrial intermembrne space	Liver, Kidney, Heart
AK3	26K	Mitochondrial matrix	Most Tissues
AK4	27K	Mitochondrial matrix	Kidney, Liver, Heart
AK5	22K	Cytosol	Brain

discuss about physiological and genetic aspects of this enzyme.

Enzymology and Protein Structure

AK catalyzes a reversible high-energy phosphoryl transfer reaction between adenine nucleotides; AMP+ATP $\rightleftarrows$ 2ADP. AK is an enzyme that is familiar to old biochemists. In 1942 Herman Kalcker with Sidney Colowick discovered this enzyme,[5] and later in 1955 Lafayette Noda and Stephan Kuby crystallized the enzyme.[6] In 1970s Dr. Noda and German biochemists including Heiner Schirmer determined the amino acid sequence of pig skeletal muscle AK.[7] In 1974 the Georg Schulz's group determined the tertiary structure by X-ray crystallography.[8]

AK is a ubiquitous enzyme and contributes to the homeostasis of cellular adenine nucleotide composition as well as the nucleotide biosynthesis. It is an essential enzyme for single-cell organisms, because it was shown that mutation in the gene is lethal in *E. coli*[9] and *Schizosaccharomyces pombe*.[10] Three isozymes that are encoded by separate genes have been characterized in vertebrates (Table 1). AK1 is present mainly in the cytosol of skeletal muscle, brain, heart and erythrocytes, while AK2 is localized mainly in the mitochondrial intermembrane space of liver, kidney and heart. Another isozyme AK3, which uses GTP instead of ATP as a phosphate donor in the reaction, is localized in the mitochondrial matrix of various tissues. Recently, two more AK isozymes, AK4 and AK5, were documented, although enzyme characterization and genetic analysis of those isozymes remain for the future study. Our group isolated cDNAs of isozymes, AK1, AK2 and AK3, and determined their genomic structures.[11–16]

Fig. 1 Participants in the US-Japan Cooperative Seminar on Metabolic Control, held at Kyoto International Conference Hall, Nov. 29–Dec. 1, 1966. Organizers, Dr. O. Hayaishi and Dr. D. E. Atkinson, are the second figure from the right and the third figure from the left in the front row, respectively. Dr. J. Monod, an invited guest, is the fifth figure from the left in the front row.

In the research from many laboratories, AK has been isolated from a variety of organisms, and the amino acid sequences were determined both from direct analysis and from gene analysis. When they are aligned, one first realizes that the amino acid sequences are similar to a great extent. Second, AKs can be divided into two groups; the long and the short forms with and without extra 27 amino acid residues in the middle portion, respectively.[17] AK1 belongs to the short

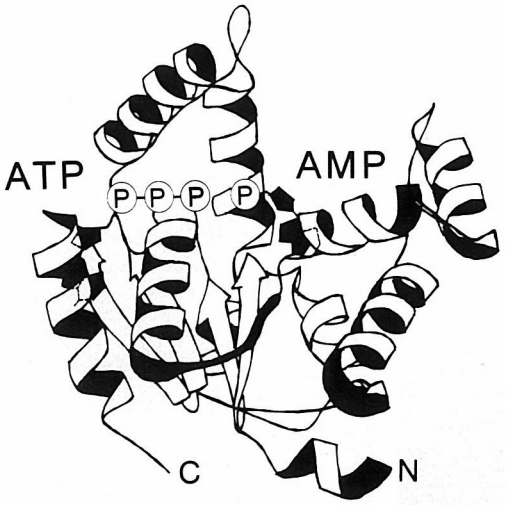

Fig. 2 Ribbon representation of tertiary structure of AK1. The binding sites for ATP and AMP are depicted.[18]

type, whereas AK2 and AK3 as well as bacterial AKs belong to the long form. The tertiary structures of the enzymes are also very similar to each other.[18] All the enzymes have a structure containing five parallel β-strands in the center of the molecule, which are connected with α-helices. The protein has three domains: i) the nucleoside monophosphate (NMP) domain, ii) the LID domain, and iii) the CORE domain. The NMP domain containing the AMP-binding site consists of two α-helices. The LID domain is located on the top of the molecule and contains four short β-strands. AK1 has a very short LID domain, because the 27-residue deletion occurs in this portion. Using di-adenosine pentaphosphate, an inhibitor of the enzyme, the binding sites for AMP and ATP were predicted (Fig. 2).[19] In the catalytic process, the LID domain is thought to move upon substrate binding in an induced fit manner toward the main body of the protein.[20] The rest of the molecule of AK called the CORE domain containing the ATP-binding site is also conserved in many species.

We cloned the gene encoding AK from *Halobacterium halobium*, an extremely halophilic bacterium[21] and *Chlamydia pneumoniae*, an obligatory intracellular parasite.[22] Both AKs have a Zn-motif consisting of four Cys in the LID domain. Such a structural Zn had previously been reported in Gram-positive bacteria. Zn in the AKs stabilizes the enzyme and probably contributes to proper folding of the protein.

AK1 Deficiency

Professor Shiro Miwa of Tokyo University reported a Japanese case of AK1 deficiency in 1983, which was the third case in the world. The patient was a 10-year-old girl at the time of discovery. Although her physical and mental development was normal, symptoms typical of hemolytic anemia were occasionally recognized such as paleness, mild jaundice and a palpable liver and spleen. Splenoectomy was performed at the age of 15. Since then she was partially relieved of these symptoms. The patient's erythrocytes had about 50% activity of AK, and it had a high Km value for ADP with a slight change in electrophoretic mobility and thermostability. From these findings, we speculated that there should be a mutation in the structural gene of the enzyme. The patient's father had normal AK activity, but her mother and grandfather's erythrocytes had about 50% activity. Therefore, the patient would be in the heterozygous state. We analyzed the genomic clones of the girl and found that the codon for the 128th Arg changed to Trp by a C to T transition on one of her chromosomes (Fig. 3).[23] The AK1 protein having this sub-

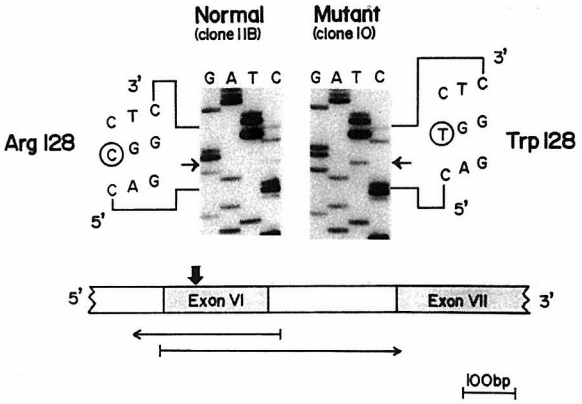

Fig. 3 Nucleotide sequencing of the normal and mutant AK1 alleles. Sequence ladders of the region containing a base substitution are shown. Exon-intron organization of the affected area is depicted in the lower part. A vertical arrow indicates the position of the base substitution.[23]

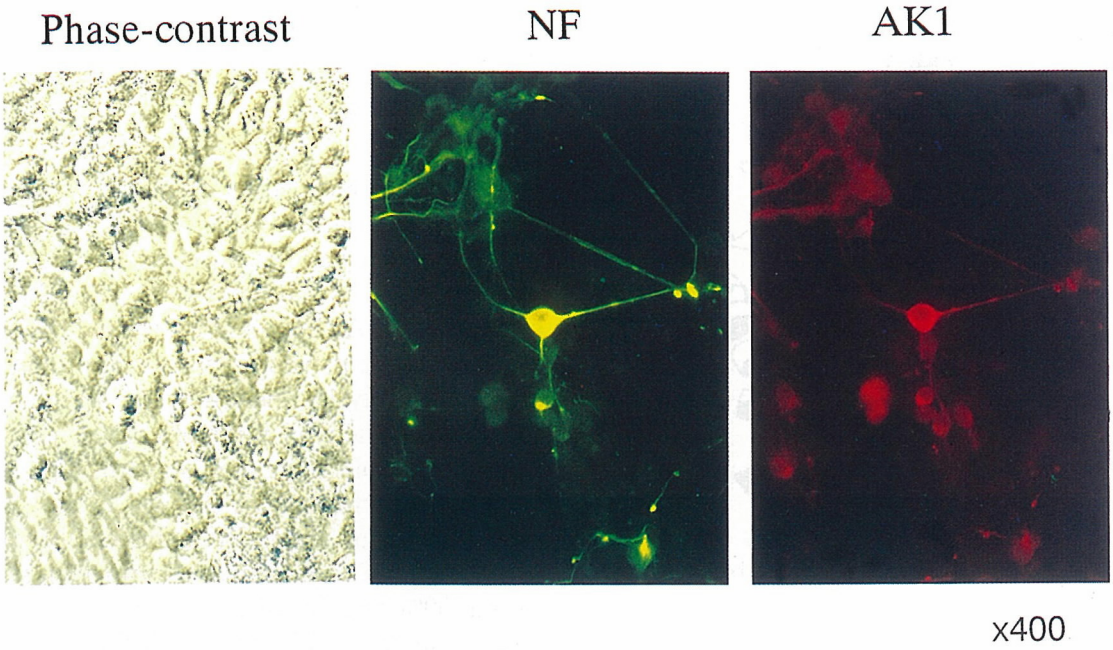

Fig. 4 Photomicrographs of differentiated P19 cells after 7 days of cultivation for differentiation. Phase-contrast (left) and double-immunofluorescent images (middle and right) of P19 cells are shown.[24] Anti-neurofilament antibody is stained in yellow, and anti-AK1 antibody, in red.

stitution was made by the site-directed mutagenesis technique. The results showed that the amino acid substitution actually affected both the solubility and the specific activity of the enzyme. The enzyme activity was as low as 1% that of the normal enzyme. The location of the substitution is near the ATP binding site. The bulky and hydrophobic side chain of Trp probably affects the binding of ATP, the conformational change during catalysis and the stability of the enzyme.

AK1 Isozyme

We focused our attention on the finding that a fairly large amount of AK1 is present in brain and hence analyzed AK1 during nerve development. P19 mouse embryonal carcinoma cells were treated with retinoic acid. After two days of cultivation, neuron-like cells started to appear, and the numbers increased. Anti-AK1 antibody strongly stained not only nerve cell bodies but also neuronal processes that were recognized by antibody against neurofilaments. In 7 days, astroglia-like cells that were stained with antibody against glial fibrillary acidic protein

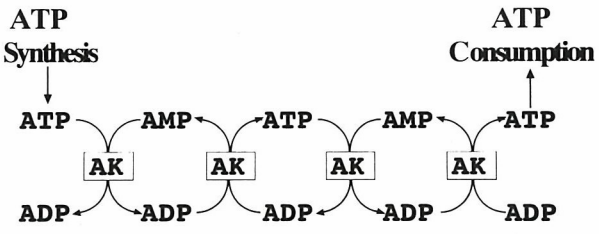

Fig. 5 A high-energy phosphoryl transfer chain involving a multiple of two AK molecules. Note that the β-phosphoryl of ADP is a high-energy phosphate that either accepts or transfers a phosphoryl from ATP or to AMP, respectively.

appeared, but they were completely different from nerve cells bearing processes that were recognized by the AK1 staining (Fig. 4.) These findings indicate that AK1 exists at high concentrations in neuronal processes of differentiated PC19 cells. In isolated cortex cells from rat brain, neuronal fibers were also clearly stained with anti-AK1 antibody. Therefore, the concentration or the accumulation of AK1 protein in neuronal processes also occurs under more physiological conditions than

tissue culture conditions.

The existence of AK1 in the neuronal process at high concentrations should be important for transferring the high-energy phosphate from energy producing sites to energy consuming sites in axons. ATP is constantly needed for the maintenance of membrane potential and axoplasmic flow in axons. In these cellular portions, there should be a high-energy phosphoryl transfer system, because it is hard to consider that active ATP-producing mechanisms are operating in the neuronal process. When AK1 molecules are set in array in the process, the γ-phosphate in ATP is transferred to the β-position of ADP by the AK1 reaction. The β-phosphate in ADP is then transferred to the γ-position of ATP by the next AK1 reaction. Thus using two AK1 molecules, the high-energy phosphate can be transmitted to the near-by location. Therefore, a multiple of two AK1 molecules forms a phosphoryl transfer chain (Fig. 5). In this way the chemical energy can be transmitted from the ATP-synthesizing site to the ATP-consuming site at a fairly remote position and thus overcome the time required for simple diffusion of ATP molecules.

AK2 and AK3 Isozymes

As described above, both AK2 and AK3 are mitochondrial proteins. They have no cleavable presequence at the N-terminus. We confirmed the submitochondrial localization of the two isozymes.[25] Rat liver mitochondria were treated with varying amounts of digitonin that loosens the outer mitochondrial membrane and subjected to proteinase K digestion. The AK isozymes were analyzed with rabbit antibody against AK2 that cross-reacts to AK3. The band of AK2 in the intermembrane space disappeared. The band of AK3 remained after the protease digestion, but it was digested when mitochondria were pre-treated with digitonin at a higher concentration to dissolve the inner mitochondrial membrane. These experiments clearly indicated that AK2 is present in the intermembrane space, while AK3 is in the matrix of mitochondria.

In our analysis with in vitro transport system using isolated rat liver mitochondria, both AK2 and AK3 required the inner membrane electrochemical potential for their import as other mitochondrial proteins. In the transport process, AK2 and AK3 competed with the adrenodoxin precursor, which is imported into the matrix through a mechanism common to other mitochondrial matrix proteins. AK3 was transported into mitochondria post-translationally, while AK2 import in vitro needed simultaneous proceeding of protein translation. Such a co-translational import mechanism of AK2 might contribute to the bi-topological distribution of this isozyme in both the cytosol and the mitochondrial intermembrane space. Further analysis is needed to determine which part of the molecules is responsible for targeting the proteins to proper mitochondrial compartments. AK3 is localized in the mitochondrial matrix. In the matrix, the citric acid cycle generates GTP at the succinate thiokinase reaction. Since adenine nucleotide translocator in the inner membrane of mitochondria has narrow substrate specificity, GTP must be converted to ATP if the high-energy phosphate is needed in the outside of mitochondria. In the biochemistry textbook, the high-energy phosphate is transferred from GTP to ADP by nucleoside diphosphate kinase. However, taking into account of the localization of AK3 and its constitutive expression, AK3 may also be involved in the transfer of the phosphate moiety from GTP to ADP, which is further phosphorylated to ATP in the oxidative phosphorylation reaction (Fig. 6).

The oxidative phosphorylation reaction is tightly controlled by ADP. The adenine nucleotide translocator provides ADP from the intermembrane space to the matrix, and at the same time it carries ATP out of the matrix. Therefore, there should be an efficient ADP-supplying system near the outer surface of the inner membrane. In liver, AK2 is localized in the intermembrane space and probably functions as an ADP-supplying system. Our analysis showed that in rat liver 50% of the total AK2 protein is present in the cytosol, while 50% of the protein is in the intermembrane space of mitochondria. If the phosphoryl transfer system consisting

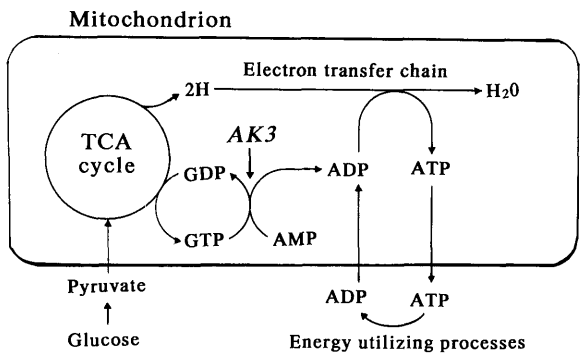

Fig. 6 A possible role of AK3 in the mitochondrial matrix.

of two molecules of AK2, one in the cytosol, the other in the intermembrane space of mitochondria, is functioning, the high-energy phosphate of ATP generated in mitochondria can be transmitted via this system to the cytosol, where it is used, in liver (Fig.7). Heart has very high AK1 activity as compared with AK2, while kidney has almost equal amounts of the AK1 and AK2 activity. In these tissues, the phosphoryl transfer system is probably constituted by AK1 in the cytosol and AK2 in the intermembrane space of mitochondria. Skeletal muscle and brain has only AK1. We now know that a portion of AK1 is also present in mitochondria of brain and kidney. Since a large amount of AK1 exists in the cytosol of skeletal muscle cell, muscle mitochondria probably hold AK1 in the intermembrane space, and so must be the case in brain. Therefore, the phosphryl transfer system is also operating in these tissues between AK1 in the cytosol and AK1 in the mitochondrial intermembrane space.

Glycolysis or glycogenolysis is the source of energy instead of oxidative phosphorylation in skeletal muscle. Since the content of AK1 in the cytosol is extremely high in skeletal muscle, the phosphoryl transfer system of AK1 can also be formed in the cytosol between the ATP-synthesizing site and the ATP-consuming site. Professor Nelson Goldberg of University of Minnesota proposed the high-energy phosphoryl transfer chain involving a multiple of two AK1 molecules in skeletal muscle.[26] It is known that skeletal muscle has another phosphoryl transfer system that uses P-creatine and creatine kinase.

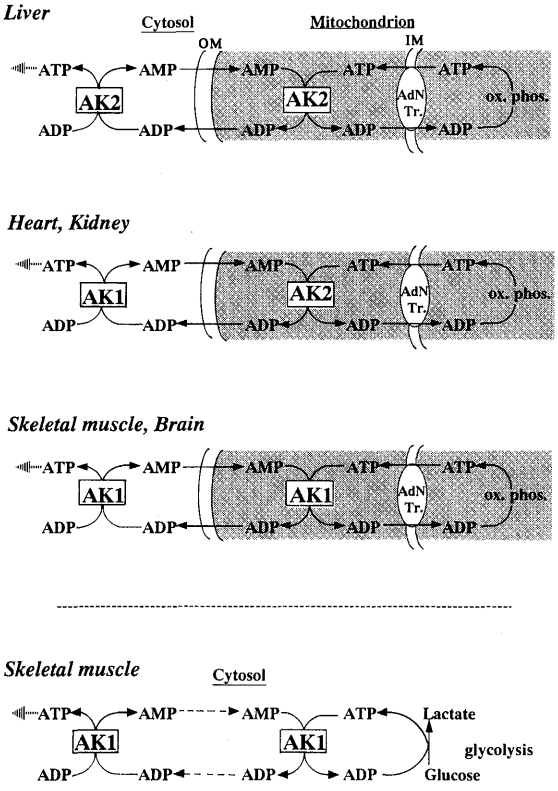

Fig. 7 Role of AK isozymes in various tissues. OM, outer membrane of mitochondria ; IM, inner membrane of mitochondria ; AdNTr., adenine nucleotide translocator ; ox. phos., oxidative phosphorylation.

Thus, these tissues are outfitted with at least two types of high-energy phosphoryl transfer system in order to cope with sudden demand of energy supply. Interestingly, the order of the amount of oxygen consumption in various tissues is skeletal muscle, brain, heart, kidney, and liver, which is exactly the same as the order listed from the bottom to the top in Fig. 7.

Evolution of AK Molecules

It is interesting to know how the AK protein family occurred in evolution. Fig. 8 is a phylogenetic tree made in collaboration with Professor Mitiko Go of Nagoya University, by analyzing the amino acid sequences of various AKs.[27] This analysis indicates that AK1, a short molecule was separated froma long AK molecule more than a billion

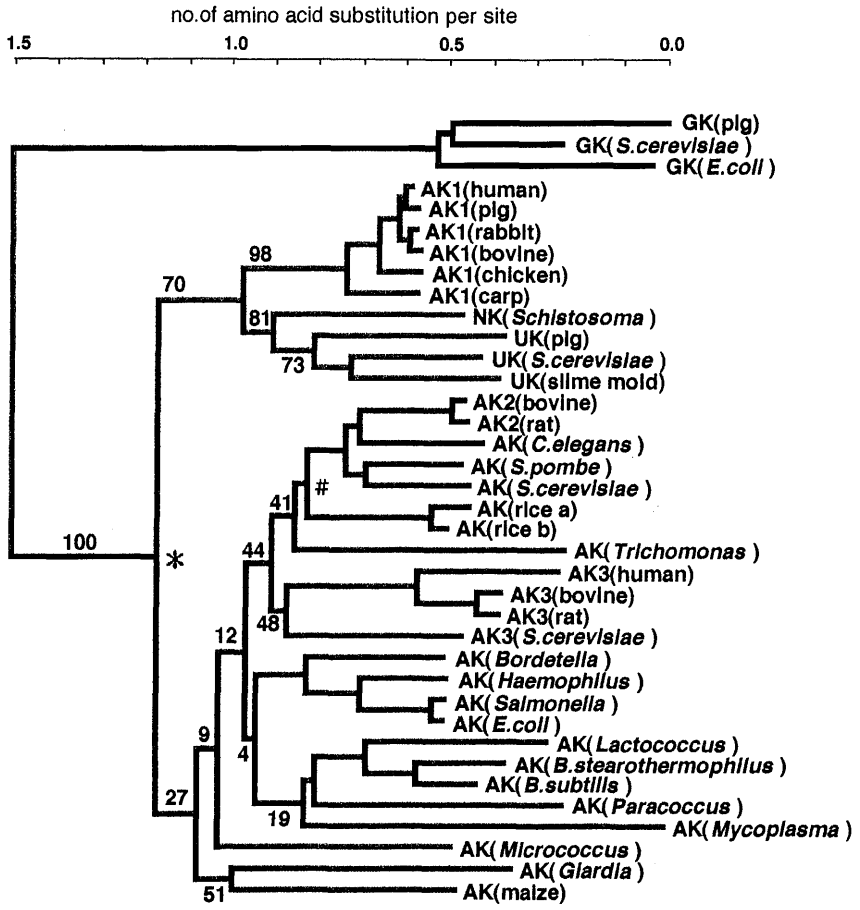

Fig 8. Phylogenetic tree of the NMP kinase family. An asterisk (*) points out the root position of the NMP kinase family tree. A sharp (#) indicates the divergence point of the eukaryotic three kingdoms in the AK2 subgroup.[27]

years ago before the separation of prokaryotes and eukaryotes. In this figure evolutionary paths of the long types are clustered at the lower part, and the short types in the upper part, respectively. AK of prokaryotes belongs to the long type, while chloroplast AK and mitochondrial AK also belong to the long type. This is consistent with the view that these organellae are the products of symbiosis of bacteria. AK3 subcluster was formed, indicating that substrate specificity for GTP was acquired after the divergence from AK2. UMP-CMP kinase is highly homologous to the short type AK. To know the branch point of long and short types, separation of GMP kinases that were clustered in the uppermost part of the figure was important.

Epilogue

AK has been known to function for the homeostasis of cellular adenine nucleotide composition as well as the nucleotide biosynthesis. The finding that AK1 is localized in the neuronal process raised the possible second regulatory function of AK, the high-energy phosphoryl transfer chain that was proposed from the experiments done in the Dr. Goldberg's laboratory. The third important aspect of AK in the metabolic regulation must be its role in the extracellular signaling. Recently, it is found that ATP and ADP are released in a regulated fashion from various cells and interact with cell surface receptors, which in turn promote a wide variety of responses in many cell types, including muscle contraction and relaxation,

vasodilation, neurotransmission, platelet aggregation, ion transport regulation and cell growth. AK may be related to the metabolism of adenine nucleotides in the extracellular signaling in the physiological and pathological processes as well as in development and differentiation of the tissues.

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