Adenosine Kinase of Arabidopsis. Kinetic Properties and Gene Expression[1]

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To assess the functional significance of adenosine salvage in plants, the cDNAs and genes encoding two isoforms of adenosine kinase (ADK) were isolated from Arabidopsis. The *ADK1*- and *ADK2*-coding sequences are very similar, sharing 92% and 89% amino acid and nucleotide identity, respectively. Each cDNA was overexpressed in *Escherichia coli*, and the catalytic activity of each isoform was determined. Both ADKs had similar catalytic properties with a K_m and V_{max}/K_m for adenosine of 0.3 to 0.5 μM and 5.4 to 22 L min^{-1} mg^{-1} protein, respectively. The K_m and V_{max}/K_m for the cytokinin riboside N^6(isopentenyl) adenosine are 3 to 5 μM and 0.021 to 0.14 L min^{-1} mg^{-1} protein, respectively, suggesting that adenosine is the preferred substrate for both ADK isoforms. In Arabidopsis plants, both *ADK* genes are expressed constitutively, with the highest steady-state mRNA levels being found in stem and root. *ADK1* transcript levels were generally higher than those of *ADK2*. ADK enzyme activity reflected relative ADK protein levels seen in immunoblots for leaves, flowers, and stems but only poorly so for roots, siliques, and dry seeds. The catalytic properties, tissue accumulation, and expression levels of these ADKs suggest that they play a key metabolic role in the salvage synthesis of adenylates and methyl recycling in Arabidopsis. They may also contribute to cytokinin interconversion.

Housekeeping enzymes fulfill basic metabolic roles that are required by all cells. The genes encoding these enzymes are often expressed constitutively although the relative level of expression may vary developmentally or in a tissue-specific manner. In many cases, these activities are represented by gene families with individual isoforms having different substrate specificities, subcellular locations, or catalytic properties. In addition, the multiple copies of housekeeping genes provide insurance against a deleterious mutation in an enzyme providing an essential cellular activity.

Adenosine kinase (ADK; EC 2.7.1.20; ATP: adenosine 5′-phosphotransferase) is a typical housekeeping enzyme that is constitutively expressed and catalyzes the phosphorylation of adenosine (Ado) to adenosine monophosphates. For catalysis, the enzyme requires the presence of a divalent metal ion, usually Mg^{2+}, and a phosphoryl donor, preferably ATP or GTP (Anderson, 1977). ADK activity was first characterized from yeast (Caputto, 1951; Kornberg and Pricer, 1951) and mammalian tissue (Caputto, 1951) where it plays a key role, along with Ado deaminase in the regulation of intracellular adenylate pools and extracellular Ado levels (Arch and Newsholme, 1978; Fox and Kelley, 1978). ADK has subsequently been characterized from other eukaryotes, including the plants lupin (Guranowski, 1979), wheat (Chen and Eckert, 1977), peach (Faye and Le Floc'h, 1997), and the moss *Physcomitrella patens* (von Schwartzenberg et al., 1998). ADK activity has not been detected in prokaryotes (Nygaard, 1983). The crystal structure of human ADK recently was determined to a resolution of 1.5 Å (Mathews et al., 1998), providing valuable insight into the substrate binding sites of the enzyme and its reaction mechanism.

ADK is involved in the salvage pathways of both adenine (Ade) and Ado and thus is a component of the adenylate metabolic network. Ade and Ado salvage activities are important for a number of reasons: (a) They prevent the accumulation of possibly inhibitory concentrations of these purines; (b) they efficiently recycle Ade and Ado into the adenylate pools; and (c) they convert cytokinin (CK) bases and ribosides to their corresponding nucleotides. Because cytokinin bases and possibly ribosides are thought to be the active forms of cytokinins, their conversion to the inactive nucleotide may be important in regulating the level of this hormone in plant cells.

Ado kinase-coding sequences have been isolated from several mammalian species including humans, rats, mice, (Singh et al., 1996; Spychala et al., 1996), the parasite *Leishmania donovani* (Sinha et al., 1999), and the moss *P. patens* (von Schwartzenberg et al.,

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1998). Here we report the isolation of two ADK genes of Arabidopsis and an initial characterization of the expression and enzymatic activities of their products. This analysis is directed toward elucidating the functional significance of ADK in plant metabolism. An understanding of how ADK contributes to housekeeping activities as well as to hormone metabolism is critical to appreciating the complexities of plant biochemistry.

RESULTS AND DISCUSSION

Isolation of Arabidopsis ADK cDNAs and Genes

Two groups of cDNAs (group I and II) were identified by screening an Arabidopsis cDNA library with the Arabidopsis expressed sequence tag (EST) Z34547 (CC10) that had regions of high sequence identity to a human ADK cDNA (Spychala et al., 1996). The largest insert from each group was sequenced along with a subsequently identified Arabidopsis EST (R30128). The EST R30128 proved to be identical to those of group I of clones. Analysis of the group II clones, which hybridized only weakly to the CC10 probe, revealed that these sequences were very similar to R30128, although small differences in their nucleotide sequences were found throughout.

The ORF of R30128 had 344 codons that began with a Met codon 49 bp downstream from the 5′ terminus and ended with a TAA stop codon 1,035 bp from the first ATG codon. The amino acid sequence of R30128 predicted a protein with a pI of 5.29 and a molecular mass of 37.8 kD, which was consistent with the molecular mass of 38 kD obtained from preliminary western analysis and within the range (25–56 kD) of previously characterized ADKs (Schomberg and Stephan, 1997). This clone contained a 216-nucleotide (nt) non-coding region at its 3′ terminus. Amino acid sequence alignment showed that the ORF of R30128 shared 56% identity with the predicted amino acid sequence of human ADK (Spychala et al., 1996) and was thus tentatively designated as *ADK1*.

Agarose gel electrophoresis analysis showed that the three group-II clones contained inserts of 2,000 to 2,200 bp. It was likely that all the clones were chimeric when their sizes were compared with the 1,300-nt transcript predicted by a preliminary northern blot. Single-pass sequence data initiated from the ends of the inserts indicated that two of the clones shared a region of nucleotide identity within their overlapping regions. Sequencing of these clones revealed that one contained 33 bp 5′ upstream of the coding region, whereas the insert of other lacked this 5′-untranslated region (UTR) as well as the first ATG codon. Each clone was associated with a different sequence that could be identified by BLAST analysis (Altschul et al., 1997; data not shown) as a non-ADK sequence. Assembly of the partial ADK sequences resulted in a 1,201-bp cDNA consensus sequence that contained an ORF of 345 amino acids with a predicted molecular mass of 37,959 D, and an estimated pI of 5.14. The reading frame ends with a TAA stop codon at position 1,036, followed by 130 bp of the 3′-UTR but lacks a poly(A) tail likely because of a cloning artifact since the 3′-UTR contains putative polyadenylation signals (AAUAAA or AAUUAA) at positions 1,107 and 1,144. This clone was designated *ADK2* and was 88% identical to *ADK1* over 1,032 nt. The sequences of both open reading frames lack identifiable transit sequences, and therefore both ADKs are likely located in the cytosol.

Corresponding genomic clones for the *ADK1* and *ADK2* cDNAs were recovered by screening an Arabidopsis genomic library with the *ADK1* cDNA, at low stringency (5× SSC, 30% [v/v] formamide, 42°C) to allow hybridization of the probe with both genes. The genes were localized within the insert by Southern analysis and completely sequenced by primer walking. The GenBank accession numbers for the *ADK1* and *ADK2* cDNA and gene sequences are AF180894, AF180895, AF180896, and AF180897, respectively.

The cDNAs were used to estimate the number of *ADK* genes in the Arabidopsis genome by Southern hybridization. Multiple DNA fragments were detected when low stringency conditions were used (5× SSC, 30% [v/v] formamide, 42°C; wash 1× SSC 42°C; Fig. 1A) with an *ADK1* probe. High stringency Southern hybridization using either the *ADK1* or *ADK2* cDNA as a probe indicated that the multiple fragments observed at low-stringency conditions could be accounted for by either *ADK1* or *ADK2* for almost all the digests tested. For example, in the *Hin*dIII digest that cleaves both cDNAs at one site, two different bands hybridized with either the *ADK1* or *ADK2* probe (Fig. 1, compare B versus C), which were equivalent to the three fragments, the smallest being a doublet, observed at low stringency (Fig. 1A). These results suggest that ADK is encoded by two genes in Arabidopsis, although there may be other ribokinases of low-sequence homology to these ADKs, which are capable of using Ado as a substrate.

Phylogenetic Analysis of ADK Sequences

Sequence comparisons between the *ADK1*- and *ADK2*-coding regions indicated that they share 89% nucleotide identity, whereas the conceptual translation products of the two cDNAs shared 92% amino acid sequence identity. Pairwise clustal analysis of these predicted amino acid sequences with ADK sequences from other sources indicated the highest identities with the plant sequences and the lowest with ADK from yeast (Table I). Queries of the GenBank EST database indicated over 800 matches to other ESTs, many of which are from other plants including *Brassica campestris*, hybrid aspen, rice, and tomato (data not shown).

The crystal structure of human ADK was recently determined to 1.5 Å resolution (Mathews et al., 1998).

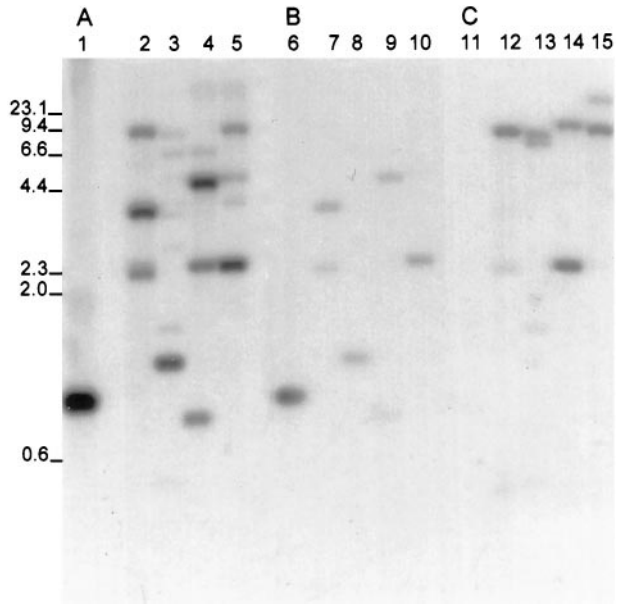

Figure 1. DNA hybridization analysis of genomic DNA with ADK probes. Arabidopsis genomic DNA (8 μg/lane) was digested with either *Hin*dIII (lanes 2, 7, and 12), *Eco*RI (lanes 3, 8, and 13), *Eco*RV (lanes 4, 8, and 14), or *Xba*I (lanes 5, 10, and 15) and the products separated by electrophoresis through a 1% (w/v) agarose gel. The DNA blots were hybridized with a radiolabeled full-length ADK1 cDNA in a hybridization solution containing either 30% (A) or 50% (B) (v/v) formamide or with the ADK2 cDNA in a 50% (v/v) formamide hybridization buffer and washed with 1× SSC at 42°C. The partial ADK1 cDNA (1 ng) was used as a positive control (lanes 1 and 6) and as a test of hybridization specificity of the ADK2 probe (lane 11). Positions of the λ*Hin*dIII fragments are shown on the left in kb.

Modeling the three-dimensional structure of the Arabidopsis isoforms using the coordinates of the human enzyme revealed a strong structural similarity between the three ADKs and none of amino acid differences between the two Arabidopsis isoforms contact the ligands ATP and Ado. Only two of the 26 residues that differ between the ADK1 and ADK2 sequences are on an exposed surface of the enzyme (Ala [187, 188] and Glu [203, 204]; [psn in ADK1, psn in ADK2]). Furthermore, all of the amino acid substitutions between the human and the plant ADKs can found in the corresponding positions of other ADK sequences (data not shown). Thus, these cDNAs code for ADKs that are very similar to each other in amino acid sequence and structurally similar to those previously described.

The sequence comparison of the *ADK* cDNAs and genes revealed that *ADK1* contains 11 introns and *ADK2* contains 10 introns. The positions of all the shared introns are conserved between the two genes (data not shown), although the length of corresponding introns differs in some cases. It is interesting that all 11 intron positions of *ADK1* are conserved in the *P. patens* ADK gene (v Schwartzenberg, P Schultz, personal communication).

The *ADK1* and *ADK2* genes were physically mapped by hybridization to filters containing the CIC (for CEPH, INRA, and CNRS) yeast artificial chromosome library (Creusot et al., 1995). These results indicated that *ADK1* maps to chromosome 3, approximately between MI467 (13.7 cM) and *APX*1b (14.7 cM), and *ADK2* is located on chromosome 5 between g3715 and CTR. Subsequently the *ADK2* region was sequenced completely by the European Union Arabidopsis sequencing project and found to be located on P1 clone MOK16 (accession no. AB005240) where it is annotated as being similar to *Zea mays* ADK.

The 5′-Upstream Regions of the ADK Genes Share Regions of High Identity

The upstream regions of the *ADK* genes are typical of other promoters that express housekeeping enzymes. These generally lack TATA and CCAAT elements commonly associated with RNA pol II promoters, and instead they often contain a high guanine-cytokine (G-C) content with one or multiple GC-rich element(s).

Only one putative transcription start site (TSS) consistent with the cap binding consensus sequence (CANPyPy) was located in *ADK1*, 87 bp upstream from the translation start codon. If the TSS at −87 is functional, the recovered *ADK1* cDNA is lacking 38 bp at its 5′ end. Unlike most TATA-less promoters, the GC composition of 5′-flanking region of the *ADK1* gene was relatively low (37%). In addition, 40

Table I. *Comparison of amino acid sequences of ADKs from various sources*

Pairwise clustal analysis of representative ADK sequences from other organisms versus the conceptual translation of the *ADK1* and *ADK2* cDNAs. Genbank accession numbers are given in "Materials and Methods."

Organism	*Z. mays*	*P. patens*	*M. musculus*	*C. griseus*	*H. sapiens*	*S. cerevisiae*	*ADK1*	*ADK2*
Z. mays	100							
P. patens	68	100						
M. musculus	58	53	100					
C. griseus	59	59	90	100				
H. sapiens	58	58	87	91	100			
S. cerevisiae	40	40	41	49	36	100		
ADK1	82	66	56	58	57	36	100	
ADK2	82	67	55	57	56	37	92	100

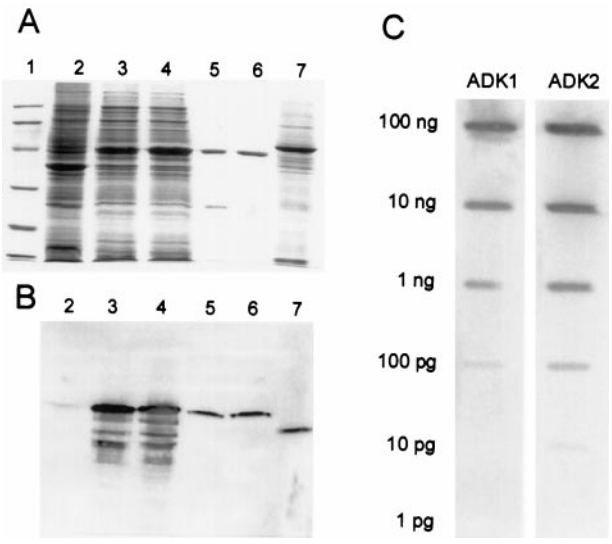

Figure 2. Isolation of ADK fusion proteins and ADK antibodies. A, Each ADK cDNA expressed as a His-tagged fusion protein in *E. coli* and purified by nickel affinity chromatography. Analysis of overexpressed ADK recombinant proteins in *E. coli* by SDS-PAGE and Coomassie Blue staining. Lane 1, Molecular mass markers in kD from largest to smallest are 97.4, 66.2, 45, 31, 21.5, and 14.4; lane 2, uninduced culture; lanes 3 and 4, induced ADK1 and ADK2, respectively; lanes 5 and 6, purified ADK1 and ADK2, respectively; lane 7, 10 μg leaf crude extract. B, Proteins from a replicate gel shown in A were transferred to PVDF and reacted with ADK antiserum. ADK breakdown products were detected in the ADK overexpressing cultures, and a 38-kD peptide was detected in the leaf tissue. C, The ADK antiserum was titered on slot blots of purified ADK1 and ADK2 containing the indicated amount of each protein.

AT-rich elements with minimum length of 6 bp were identified in the 2,378-nt upstream of the *ADK1*-coding region (data not shown). The function of the AT tracts in plant promoters is unclear since they have been documented to have either positive or negative effects on transcription depending upon the promoter under study (Tjaden and Coruzzi, 1994). There are two putative cap binding sites in *ADK2* promoter located at 24 and 43 nt upstream of the ATG start codon. This promoter has a relatively high GC content (46%) and harbors a sequence, GGCG-GCGC, similar to a GC box, −78 to −85 from the TSS at −43.

A dot-plot analysis of the sequences upstream of the start sites of translation of *ADK1* versus *ADK2* revealed two major regions of similarity located approximately −2,000/−1,310 to −1,724/−1,026 and −1,180/−979 to −832/−634 (position in *ADK1*/position in *ADK2* relative to ATG). The first region is the stronger match of the two upstream sequences (88% identity over 117 nt versus 83% identity over 129 nt). The two stretches of similarity are very close in *ADK2* (126 bp separating them), whereas they are 474 bp apart in *ADK1*. No significant matches to these sequences were found in GenBank, nor were the ends flanked by obvious direct or indirect repeat sequences. The length of these conserved sequences and their high degree of identity suggest that they may have a functional significance, although this remains to be shown.

Sequences upstream of *ADK1* and *ADK2*-coding regions were compared by pairwise BLAST analysis with the upstream non-coding regions of other Arabidopsis housekeeping genes involved in Ade metabolism, including APT1, APT2, and the de novo purine synthesis gene PurM. A stretch of 48 nucleotides, located at positions −725 to −677 from the translation start codon, was found to have 60% identity with the sequence of −347 to −396 of the *APT1* promoter (Moffatt et al., 1994) and 37% identity with the sequence at −391 to −440 of the *APT2* (Schnorr et al., 1996). A similar sequence was also found at 329 to 364 bp upstream of the coding region of the *Pur* M gene, which encodes an enzyme of the purine de novo pathway (Senecoff and Meagher, 1993). A second region positioned at −1,509 to −1,557 of *ADK1* has 64% identity with 48 nt of the *APT1* promoter located −309 to −358 from its translational start codon. These sequences might be relevant for the regulation of transcription in these housekeeping genes, but their functional significance remains to be determined.

Both ADKs Metabolize Ado and CK Substrates

The coding region of each ADK cDNA was overexpressed with a His tag in an *E. coli* host and purified by nickel affinity chromatography (Fig. 2, A and B), and the catalytic activity of each isoform was characterized. The rate of incorporation of Ado into AMP was monitored across the pH range of 6 to 9.5. Both isoforms reacted differently to each buffer system, although the trend within each buffer pointed to a pH optimum of approximately 8.0 for ADK1 and 9.5 for ADK2 (data not shown). These pH preferences are at the high end of those reported for ADK from other sources (pH 4.6–9.3; Schomberg and Stephan, 1997), but are similar to those found for ADK from peach (pH 8.5–9.5; Faye and Le Floc'h, 1997) and wheat germ (pH 7.2–8.2; Chen and Eckert, 1977). Assuming that these ADKs are cytosolic, as their sequences suggest, they may not be working at their optimum rates in vivo or other modifiers may affect their activities. The optimal ATP:$MgCl_2$ molar ratio was found to be 4:1 (Fig. 3A), and the optimal concentration of ATP was found to be 4 mM (Fig. 3B). This ATP:$MgCl_2$ molar ratio is consistent with the 4:1 and 5:1 ratios reported for other plant ADKs (Guranowski, 1979; Faye and Le Floc'h, 1997; von Schwartzenberg et al., 1998).

The K_m and V_{max} of ADK1 and ADK2 for three substrates are presented in Table II. (These results are based on the data presented in Fig. 3C.) Based on their K_ms both isoforms have a high affinity for Ado (K_ms of 0.5 and 0.3 μM for ADK1 and ADK2, respectively), but the maximal velocity of ADK2 is approx-

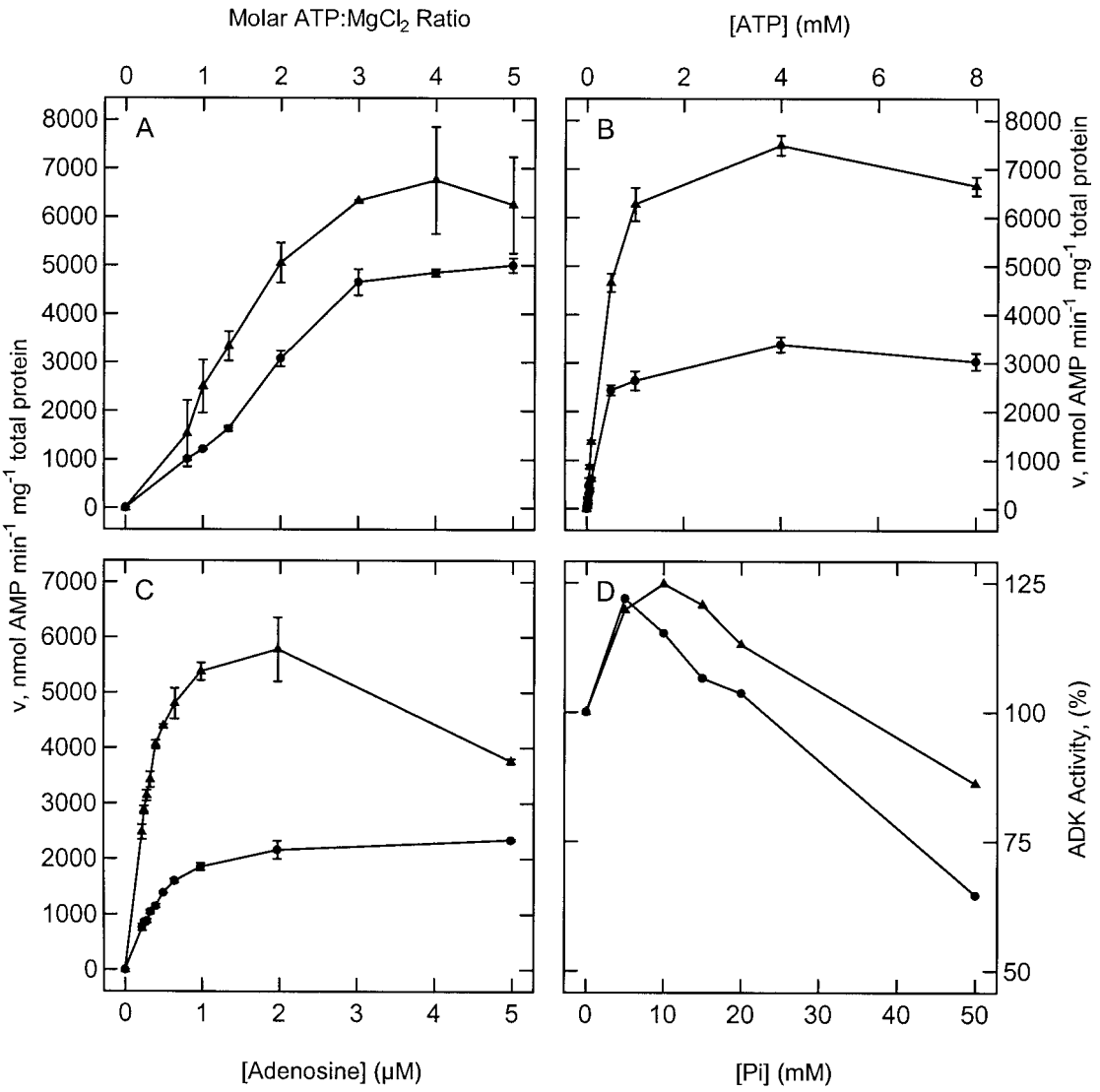

Figure 3. Kinetic analysis of ADK1 and ADK2. Results are based on the radiochemical assay using purified His-tagged ADKs as outlined in "Materials and Methods." In all panels ADK1 and ADK2 are represented by ● and ▲, respectively. A, Determination of the optimal ATP:$MgCl_2$ by varying the ratio from 0/8:1 to 5:1 while maintaining 4 mM ATP. B, ADK activity in the presence of 1 to 8 mM ATP while maintaining the 4:1 ATP:$MgCl_2$ ratio. C, Ado concentration was varied from 0.22 to 5 μM. D, Using the optimal assay conditions, the concentration of Pi was increased from 0 to 50 mM. Activity is expressed as the percentage of the activity in the absence of added Pi.

imately twice that of ADK1. The estimated intracellular concentration of Ado in plants (1–50 μM; Wagner and Backer, 1992) is above the K_m of these ADKs (0.3–0.5 μM), suggesting that they are saturated by this substrate and may be working near their V_{max}, assuming sufficient Mg:ATP is available. A comparison of the V_{max}/K_m values of ADK1 and ADK2 shows that ADK2 has a 4-fold higher efficiency than ADK1 using Ado as substrate. These catalytic parameters are quite similar to those found for other ADKs including those isolated from the plants *Lupinus luteus*, peach tree buds, and wheat germ, which had K_ms in the range of 1.5 to 8.7 μM. (Chen and Eckert, 1977; Guranowski, 1979; Faye and Le Floc'h 1997). Moreover, the V_{max} values are also consistent with those reported for purified ADKs from other sources (Schomberg and Stephan, 1997). ADK2 activity is sensitive to substrate inhibition at concentrations of Ado above 2 μM, whereas no substrate inhibition of ADK1 was observed at the Ado levels tested (Fig. 3C). Peach tree ADK is also subject to inhibition by Ado although only at Ado levels above 50 μM (Faye and Le Floc'h, 1997).

Both ADK isoforms appear to have an equal affinity for ATP (K_m = 350–370 μM; Table II; Fig. 3B), very similar those for the ADKs isolated from peach tree buds and lupin (Guranowski, 1979; Faye and Le Floc'h, 1997). These K_ms are below or of the same

Table II. *Kinetic analysis of ADK1 and 2*

Purified recombinant ADK1 and 2 were used to determine both K_m and V_{max} for three substrates of adenosine kinase. V_{max}/K_m is presented here as a measure of overall enzyme efficiency for each substrate. Assays were as described in "Materials and Methods."

Adenosine Kinase	Substrate	K_m	V_{max}	V_{max}/K_m
		μM	μmol min^{-1} mg^{-1} protein	L min^{-1} mg^{-1} protein
ADK1	Adenosine	0.50	2.7	5.4
	ATP	350	3.5	0.010
	Isopentyladenosine	3.2	0.068	0.021
ADK2	Adenosine	0.30	6.7	22
	ATP	370	7.8	0.021
	Isopentyladenosine	4.8	0.66	0.14

order of magnitude as estimates of the in vivo intracellular levels of ATP (0.5–3.2 mM) in different plants (Stitt et al., 1982; Bligny et al., 1990; Kubota and Ashihara, 1990; Gout et al., 1992). Thus Mg:ATP is probably not limiting to ADK activity, in general. However, as noted by Faye and Le Floc'h (1997), changes in energy charge or Ade nucleotide pools during development may affect ADK activity.

Two earlier studies have implicated ADK in the metabolism of CKs (Chen and Eckert, 1977; Faye and Le Floc'h, 1997). A semipurified preparation of ADK from wheat germ was reported to have a K_m for [9R]iP of 31 μM and a V_{max} of 0.0083 mol min^{-1} mg^{-1} protein. The conversion Ado to AMP by a homogeneous preparation of ADK from peach tree flower buds was reduced 40% in a competitive manner by the presence of equimolar amounts [9R]iP, suggesting that [9R]iP may be a substrate for this ADK. Similar assays of the Arabidopsis ADKs indicate that they also bind [9R]iP with approximately 10-fold lower affinity than Ado. The K_ms of ADK1 and ADK2 for [9R]iP are essentially the same (Table II; 3.2 versus 4.8 μM) but ADK2 has a V_{max} using [9R]iP that is 10-fold higher than that of ADK1. Given that the physiological concentrations of endogenous CKs in Arabidopsis are approximately 10^3-fold lower than these apparent K_m values (Åstot et al., 1998) and that the V_{max}/K_m values for [9R]iP are 150- to 450-fold lower than those for Ado, these in vitro kinetic results suggest that CKs are not the primary in vivo substrates of Arabidopsis ADK1 and ADK2. However, their low K_m values for CKs indicate they may contribute to the metabolism of CK ribosides to some extent.

As shown in Figure 3, Arabidopsis ADKs are stimulated approximately 25% by low inorganic phosphate (Pi) (5–10 mM) and inhibited at higher concentrations (50 mM). This increase of ADK activity by Pi is relatively modest compared with the 20-fold increase reported for the ADKs isolated from mammals (Gupta, 1996; Maj et al., 2000). It is hypothesized that Pi facilitates the binding of Ado to the enzyme or the formation of an enzyme-ATP-Ado complex (Hao and Gupta, 1996; Maj et al., 2000). Although cytosolic Pi concentration is difficult to measure accurately due to difficulties in distinguishing between the vacuolar, organellar, and cytoplasmic Pi pools it has been estimated to be below 1 mM (Bligny et al., 1990). Assuming these in vitro assays reflect the in vivo situation these results suggest that Pi levels would not affect ADK activity in the cytosol.

ADKs Are Constitutively Expressed to Different Levels in Various Organs

The steady-state levels of ADK1 and ADK2 transcripts in various organs were investigated by RNA blotting. Total RNA was isolated from Arabidopsis leaves, flowers, roots, and stems of 4-week-old plants. Replicate RNA blots were hybridized with gene-specific RNA probes prepared from the 3′-noncoding region of each cDNA (Fig. 4).

Both genes were transcribed in all organs tested, although the mRNA level in each varied with *ADK1* transcripts being more abundant than *ADK2* in all cases. *ADK1* was low in leaf, with higher levels in flower, stem, and root. *ADK2* expression was highest

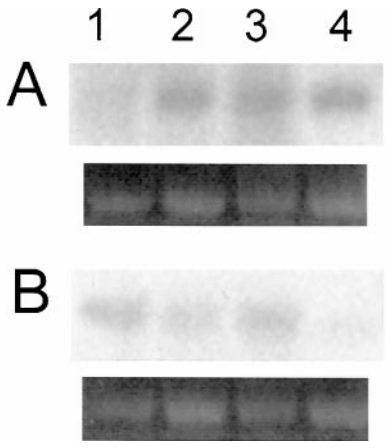

Figure 4. Northern analysis of ADK transcript levels in different organs. RNA was extracted and analyzed by northern hybridization using radiolabeled gene-specific probes for ADK1 (A) and ADK2 (B) as described in "Materials and Methods." Ethidium bromide-stained ribosomal RNA is shown below each panel. Samples were isolated from leaf (lane 1), flower (lane 2), stem (lane 3), and root (lane 4).

in leaf and stem with lower levels in flower and root. Similar results were obtained by reverse transcriptase (RT)-PCR analysis of these transcripts except that ADK1 transcript levels in leaves were higher than seen on the northern blot (data not shown). The constitutive expression pattern of *ADK1* and *ADK2* mRNAs is consistent with the classification of ADK as a housekeeping enzyme and the requirement of all cells for Ado salvage.

Purified ADK1 (Fig. 2) was used to induce polyclonal antibodies that were affinity purified and found to bind specifically to a polypeptide band of 38 kD, the expected molecular mass of Arabidopsis ADK (Fig. 2). Due to the high amino acid conservation between the two ADK isoforms, the antiserum reacted with both ADK1 and ADK2, detecting 100 pg of purified ADK1 and 10 pg of ADK2 when used at a 1:5,000 dilution (Fig. 2).

This serum was used to monitor the relative level of ADK protein in various organs by immunoblotting. There was substantial ADK accumulation in all organs with the highest levels found in silique, stem, and mature flowers and lower amounts in leaves, roots, and dry seeds (Fig. 5). ADK protein accumulation increased in flowers from 6-week- versus 4-week-old plants and decreased in leaves from 6-week- versus 3-week-old plants. The ADK protein accumulation generally reflected the mRNA levels: consititutive presence of ADK with the lowest levels in leaves and dry seeds (not shown) and higher levels in stems and flowers.

ADK catalytic activity was determined for the individual extracts used for the western blots. The highest ADK activity was detected in stems and flowers. Somewhat lower levels were found in leaves, decreasing as the plant aged. Dry seeds, roots, and siliques contained low, but detectable levels of ADK. The relative enzyme activity in the organs tested reflects the relative ADK protein accumulation in stems, leaves, and flowers but not in roots, siliques, and dry seeds, which have very low enzyme activity but apparently substantial ADK protein levels. Addition of an extract of dry seed or roots to a stem extract lowered the expected ADK enzyme activity in stem extracts dramatically, suggesting that root and dry seed extracts contain an inhibitor of ADK activity that is not removed by the desalting step used to prepare the extracts. The identity and significance of the inhibitor, which is also heat stable (M. Allen, unpublished results), remains elusive. Leaf enzyme activity was also lower than might be expected based on the immunoblot, although the difference was not as great as that found for root, silique, and dry seed. The immunoblots measuring ADK protein level and the assay are not specific for the two isoforms, which do differ somewhat in their reaction rates and catalytic efficiencies. The relative levels of the two isoforms may differ in some organs such as leaf and root based on northern analysis and

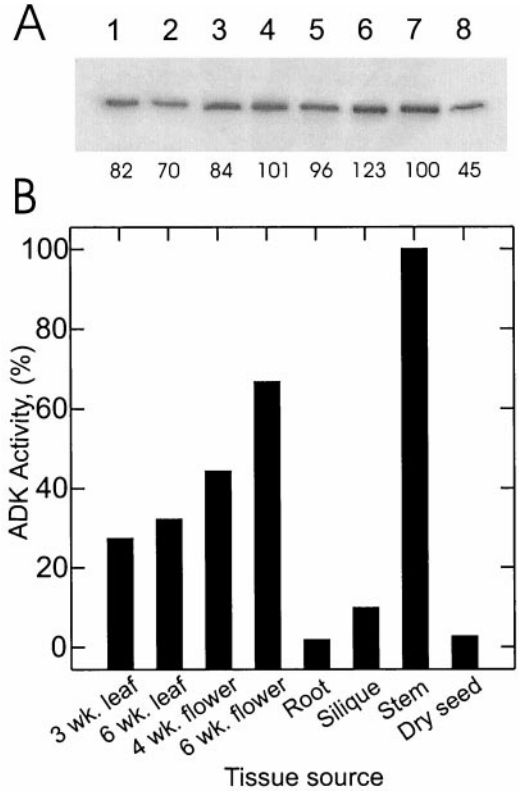

Figure 5. Analysis of ADK protein levels in various organs of Arabidopsis. A, Ten micrograms of total protein from crude extracts prepared from leaves of 3- and 6-week-old plants (lanes 1 and 2), flowers of 4- and 6-week-old plants (lanes 3 and 4); roots (lane 5); siliques (lane 6); stems (lane 7); and dry seeds (lane 8) was separated by SDS-PAGE. Each sample was prepared from organs collected from a pool of 10 plants. ADK detected using a fluorescent substrate, quantified, and expressed as a percentage of the amount detected in stems is indicated below each lane. B, The same extracts analyzed in A were desalted and assayed for ADK activity using the radiochemical assay described in "Materials and Methods." Activity is expressed as a percentage of total ADK activity in stem tissue (18.9 nmol mg^{-1} min^{-1}).

RT-PCR. Thus, differences in the relative abundance of the two isoforms may contribute to some of the observed differences between activity and protein levels. The relative ADK activity in the organs tested does not reflect the pattern expected for an enzyme involved in CK biosynthesis, assuming the in vitro assays reflect in vivo activity levels. For example, roots are thought to be the site of CK biosynthesis yet they have very low ADK activity. Thus, it is unclear at this time how much of a contribution ADK makes to CK biosynthesis.

The constitutive expression pattern and high catalytic activities of these ADKs are consistent with their primary role being the salvage synthesis of adenylate nucleotides from Ado, the predominant source of which is the transmethylation cycle. Lack of ADK activity could theoretically lead to an increase in Ado and a reduction in *S*-adenosyl-Met-dependent transmethylation (Poulton, 1981). Further characterization

of the subcellular locations, and tissue and developmental expression patterns of these ADKs will be important to understanding their contributions to the maintenance of adenylate and methyl recycling as well as their involvement in cytokinin interconversion.

MATERIALS AND METHODS

Seed Material and Germination

Arabidopsis (ecotype Columbia) seeds were suspended in 0.1% (v/v) agar and sown in plastic pots (10 cm deep) containing a 50:50 mix of "Sunshine LC1 Mix" and "Sunshine LG3 Germination Mix" (JVK, St. Catharines, ON) that had been thoroughly drenched with water and allowed to drain for 30 min. The plants were grown at 19°C with a day length of 16-h fluorescent illumination at 130 to 150 μmol m^{-2} s^{-1} photosynthetically active radiation in a growth chamber.

Sterilized seeds were sown in Petri dishes containing Murashige and Skoog solid medium prepared from Murashige and Skoog salts mixture (Sigma, St. Louis), supplemented with 2.56 mM MES [2-(*N*-morpholino)ethanesulfonic acid], 30 g/L of Suc, pH 5.7 to 5.8, and 15 g/L of agar. The seeds were stored at 4°C in the dark for at least 12 h to synchronize germination; they were then transferred to a growth cabinet at 19°C, under continuous fluorescent light (100 μmol m^{-2} s^{-1} photosynthetically active radiation).

Library Screening and Gene Mapping

Initially, the Arabidopsis EST database was searched for sequences similar to the human ADK amino acid sequence (U33936). One clone (CC10, EST accession nos. Z34547 and Z34146), which shared regions of 61% (39/63) and 60% (12/20) identity to the human ADK sequence, was found. Subsequent sequencing of CC10, and comparison of its predicted amino acid sequence with the human ADK sequence indicated that CC10 is a partial *ADK* cDNA clone with a size of 955 bp.

A Lambda ZAP II (Stratagene, La Jolla, CA) cDNA library (containing sized cDNA inserts of 1–2 kb) of Arabidopsis (ecotype Columbia) was obtained from the Arabidopsis Biological Resource Centre at Ohio Stock Center (Gubler and Hoffman, 1983; Kieber et al., 1993). Approximately 2×10^5 recombinant phages were screened by plaque hybridization using a radiolabeled CC10 probe.

An Arabidopsis (ecotype Columbia) genomic library, containing inserts of 5 to 20 kb ligated into the *Eco*RI and *Xba*I sites of λGEM11 (Promega, Madison, WI) made by Drs. John Mulligan and Ron Davis (Stanford University) was kindly provided by Dr. Philip Guerche (Institut National de la Recherche Agronomique, Versailles, France). Approximately 6×10^4 phage were grown on *Escherichia coli* LE392 host cells, transferred to nitrocellulose membranes, and probed with the radiolabeled *ADK1* cDNA (R30128) in a 30% (v/v) formamide hybridization buffer.

All putative *ADK* genomic phage were digested with either *Eco*RI, *Sac*I, *Xba*I, or *Xho*I and analyzed by gel electrophoresis. The *Eco*RI fragments (two 1.3 kb and 2.3 kb) of the *ADK1* genomic phage clone and the 3-kb *Xho* fragment of the *AK2* genomic phage DNA, which hybridized to their corresponding cDNA probes, were used for subcloning. The plasmid pZErO (Invitrogen, Carlsbad, CA) was used as the subcloning vector for genomic phage clones.

Each ADK gene was mapped by hybridization to filters containing an Arabidopsis yeast artificial chromosome library (Creusot et al., 1995). The filters were generously provided by C. Camilleri and D. Bouchez (Institut National de la Recherche Agronomique), and they interpreted the hybridization results.

Sequencing of Arabidopsis *ADK* cDNAs

Sequencing of the cDNAs was performed by the dideoxynucleotide chain termination method, using Sequenase version 2.0 as suggested by the manufacturer (United States Biochemical, Cleveland) and sequence-specific primers. Both *ADK1* and *ADK2* gene subclones were sequenced by The Institute for Molecular Biology and Biotechnology (McMaster University, Hamilton, Canada). All data were either gathered twice or obtained from both strands of DNA.

Sequence Data Analysis

Eighteen ADK amino acid sequences from other sources were recovered from the National Center for Biotechnology Information integrated databases using the Entrez browser (Benson et al., 1999) or by BLAST analysis of GenBank (Altschul et al., 1997). The ADK sequences in the data set and their GenBank accession numbers are as follows: *Cricetulus griseus*, P55262; *Homo sapiens*, U33936.1; *Mus musculus*, P55264; *Physcomitrella patens*, Y15430.1; *Saccharomyces cervisiae*, P47143; and *Zea mays*, AJ012281.1. Pairwise alignments were performed using Clustal W (version 1.7). The Arabidopsis ADK amino acid sequence was threaded into the atomic structure of the human ADK using RasMol version 2.4 (Glaxo Wellcome, UK).

DNA- and RNA-Blot Analysis

Genomic DNA was isolated from the leaves of 4- to 6-week-old plants as described by Leutwiler et al. (1984). Eight to 10 μg of genomic DNA was digested overnight with *Eco*RI, *Eco*RV, *Hin*dIII, or *Xba*I in a total volume of 100 to 150 μL according to standard procedures. DNA blots were hybridized with ^{32}P-labeled probes of the *ADK1* or the *ADK2* cDNA. The blots were prehybridized and hybridized at 5× SSC, 30% to 50% (v/v) formamide, at 42°C. Autoradiography was performed at −80°C using X-OMAT-AR film (Eastman-Kodak, Rochester, NY) with two intensifying screens (DuPont, DE).

Total RNA was isolated from flowers, leaves, and roots of Arabidopsis following the procedure described by Logemann et al. (1987). The method of Vicient and Delseny (1999) was used to extract RNA from siliques and dry seed. RNA was transferred to nylon membranes (Hybond N^+,

Amersham, Buckinghamshire, UK) and incubated in hybridization buffer at 65°C with ^{32}P-labeled gene specific RNA probes prepared by in vitro transcription of terminal regions of each cDNA (nt 1,047–1,240 for *ADK*1 and nt 1,040–1,195 for *ADK*2), which had been subcloned into Bluescript KS (Stratagene, CA). The blots were washed to final stringency of 0.1× SSC, 0.1% (w/v) SDS at 65°C for 30 min. Bound probe was detected using a Storm 860 Phosphoimager (Molecular Dynamics, Sunnyvale, CA) following an 8-d exposure to the storage phosphor screen.

Overexpression of ADKs

The coding region of each *ADK* cDNA was cloned into the *Nco*I-*Bam*HI sites of pET30a (Novagen, Madison, WI) yielding the recombinant plasmids designated 330-16 (*ADK1*) and 331-4 (*ADK2*), for expression of each ADK in BL21(DE3) pLysS. It was not possible to maintain the pET vector carrying either *ADK* cDNA in the absence of pLysS. ADK expression was induced in cultures grown at 37°C to an A_{600} of 0.6 by the addition of 1 mM isopropylthio-β-galactoside, followed by incubation for an additional 5 h. The majority of the ADK was in the soluble fraction and was recovered by chromatography on a nickel column according to the manufacturer's instructions (Novagen).

Generation and Affinity Purification of ADK Antiserum

Purified ADK1 was used to induce antibodies in New Zealand White female rabbits. Seven hundred micrograms of pure ADK1 (bearing the His tag) was mixed with Freund's complete adjuvant and injected subcutaneously in two sites of the shoulder of one rabbit. Two booster shots of 350 μg of ADK1 emulsified with Freund's incomplete adjuvant were given 3 and 6 weeks later. Serum was collected after the first boost and affinity-purified by passage over an ADK-affinity column (Gu et al., 1994). The purified serum specifically detected 100 pg of pure ADK1 or 10 pg of ADK2 used at a dilution of 1:5,000 on immunoblots.

Immunoblots

Proteins extracted in HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid] buffer as described for the ADK enzyme assay were separated by electrophoresis through 12.5% (v/v) SDS polyacrylamide gels. Ten micrograms of total protein for each sample was applied to each lane. Protein concentrations were estimated by the method of Bradford (1976) using bovine serum albumin as the standard. Following SDS-PAGE, gels were equilibrated in transfer buffer (39 mM Gly, 48 mM Tris-HCl, pH 8.7, 0.0375% [v/v] SDS, 20% [v/v] methanol) for 15 min and electrophoretically transferred to a polyvinylidene difluoride (PVDF) membrane using a semidry transfer apparatus (Bio-Rad Laboratories, Hercules, CA) for 30 min at 20 V. After air-drying, transfer efficiency was monitored by staining the blot with 0.2% (w/v) Ponceau S in 3% (w/v) trichloroacetic acid for 10 min. The PVDF membrane was incubated for 30 s in 1 μg/mL polyvinyl alcohol (M_r 30,000–70,000; Sigma) rinsed in water and then incubated overnight in 10 mM NaH_2PO_4, 150 mM NaCl, pH 7.2, 0.3% (v/v) Tween 20, 1% (w/v) Carnation dry milk powder containing affinity-purified polyclonal anti-ADK antibodies diluted 1:5,000. After a 5-min wash and 3 × 10-min washes with the same buffer, the membrane was then incubated with a 2,000-fold dilution of alkaline phosphatase-conjugated goat anti-rabbit IgG (Sigma). The membrane was washed as for the primary antibody, and bound antibody was detected by reaction with 10 μL cm^{-2} ECF (Amersham-Pharmacia Biotech, Uppsala) and quantified using a Storm 860 phosphorimager.

Plant Protein Preparation

Fresh plant tissue (approximately 100 mg) was ground thoroughly with a hand-held glass homogenizer in 50 mM HEPES buffer (pH 7.2) at a ratio of 1 mL for 250 mg fresh weight. Cellular debris was removed by two sequential 2-min centrifugations at 14,000*g* at 4°C, removing the supernatant to a new tube each time. To remove any small molecules including nucleosides and nucleotides from the crude extract, 100 μL of supernatant was passed through a 1.2-mL Sephadex G25 (medium grade, Amersham-Pharmacia Biotech) column prepared in a microfuge tube. Eluant was collected after a 15-s centrifugation step at full speed in a table-top clinical centrifuge.

ADK Enzyme Assay

The ADK assay measures the conversion of radioactive Ado to its corresponding monophosphate derivative in the presence of ATP and $MgCl_2$. The products of the reaction were verified by HPLC (Moffatt et al., 1991; data not shown). ADK activity was determined in a total reaction volume of 50 μL, which contained 3 μL of diluted crude extract or 3 μL of diluted His-tag purified protein, 50 mM HEPES-KOH buffer (pH 7.2), 1 mM $MgCl_2$, 4 mM ATP, 2 μM [2,8-^{3}H]Ado (57 mCi mmol^{-1}; ICN, CA) or ^{3}H-isopentenyladenosine (^{3}H-[9R]iP [666 GBq mmol^{-1}] synthesized according to Laloue and Fox, 1987)), 0.5 mg/mL bovine serum albumin, and 30 mM NaF. For assays of ADK activity in crude extracts, 2.5 μM deoxycoformycin (Warner Lambert), an inhibitor of Ado deaminase, was added. The reaction was incubated for 5 min at 30°C and then stopped by the addition of 1 mL of ice-cold stop buffer (0.05 M NaOAc, 2.0 mM K_2HPO_4, pH 5.0), followed by precipitation of the AMP with 200 μL of ice-cold 0.5 M $LaCl_3$. Assays using ^{3}H-Ado were left on ice for at least 30 min, and the product was collected by vacuum filtration through a glass-fiber filter with a pore size of 1.2 μm (Enzo Diagnostics, NJ). The filter was immersed in 4 mL Cytoscint (ICN) for at least 1 h prior to quantification of bound nucleotide by liquid scintillation counting (model LS 1701, Beckman Instruments, Fullerton, CA). Each determination was performed in triplicate. Assays with ^{3}H-[9R]iP as substrate were stopped by the addition of 250 μL of ethanol and the conversion to the corresponding nucleotide was quantified

by HPLC (Moffatt et al., 1991). Assays using both the His-tag purified proteins and crude extracts were linear over a 10-min incubation period. His-tag purified protein assays were linear with respect to enzyme added from 0.1 ng reaction^{-1} to 2 ng reaction^{-1} (0.3–0.6 ng were routinely used in each assay); crude plant extracts were found to be linear from 0.1 to 1.0 μg of total protein added to each reaction.

The activity levels of ADK1 and ADK2 were examined from pH 6 to 9.5. Four different buffer systems were used: 2-[*N*-cyclohexylamino] ethanesulfonic acid (pH 9.0 and 9.5; Sigma); HEPES (pH 7.0, 7.5, 8.0, and 8.2; Sigma); piperazine-*N*,*N*′-bis[2-ethanesulfonic acid] (pH 6.0 and 7.0; Sigma); and *N*-tris[hydroxymethyl]methyl-3-aminopropanesulfonic acid (pH 8.0, 8.5, and 9.0; Sigma). The levels of ADK activity differed in each of these four buffers, as well as in the buffers 3-[*N*-morpholino]propanesulfonic acid and Tris. HEPES was chosen as the final assay buffer because ADK had the highest V_{max} in this buffer and the activity remained linear for at least 10 min. Optimal ATP and $MgCl_2$ levels were determined using the standard assay conditions, while varying the ATP level and ATP:$MgCl_2$ ratio until maximum reaction activity was attained. The affect of Pi concentration on ADK activity was studied by adding K_2HPO_4 to a final concentration of 0, 5, 10, 15, 20, or 50 mM.

Statistical Analysis and Kinetic Calculations

The enzyme kinetic parameters of K_m, V_{max}, and V_{max}/K_m were estimated from the radiochemical assay data using Systat 8.0 (SPSS, Chicago). Data gathered were fitted to the Michaelis-Menten equation using non-linear regression, which allowed the estimation of K_m and V_{max} directly. K_m is a measure of affinity of an enzyme for a substrate; V_{max} is the maximal enzyme reaction rate; V_{max}/K_m is a measure of overall enzyme efficiency.

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