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APS Kinase from *Arabidopsis thaliana:* Genomic Organization, Expression, and Kinetic Analysis of the Recombinant Enzyme

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The gene encoding 5′-adenylylsulfate (APS) kinase (EC 2.7.1.25) (*APK*) was cloned from *Arabidopsis thaliana*. There is a single *APK* locus in *A. thaliana*. The coding sequence of the gene is composed of 7 exons, interrupted by 6 introns. A transcriptional initiation site was detected 120 bp 5′ of the initiation codon. *APK* mRNA is slightly more abundant in leaves than in roots of *A. thaliana* and its level does not change in response to sulfur starvation. The *APK* protein, synthesized in vitro, is able to enter isolated intact chloroplasts. Recombinant APS kinase shows maximal activity at 10 μM APS with 5 mM ATP, but it is inhibited at APS concentrations above 10 μM. The inhibition is alleviated at higher ATP concentrations. Reciprocal plot analysis showed that the theoretical V_{max} is ~1.2 μmol min^{-1} mg^{-1} at 25°C, pH 8.0; the K_m values are 3.6 μM APS and 1.8 mM ATP.

The assimilation of sulfate by living organisms is initiated by formation of 5′-adenylylsulfate (APS) catalyzed by ATP sulfurylase (EC 2.7.7.4) (reaction 1). APS kinase (EC 2.7.1.25) phosphorylates APS to 3′-phosphoadenosine 5′-phosphosulfate (PAPS) (reaction 2).

$$SO_4^{2-} + ATP \rightarrow APS + PPi \text{ (reaction 1)}$$

$$APS + ATP \rightarrow PAPS + ADP \text{ (reaction 2)}$$

ATP sulfurylase catalyzes an unfavorable reaction with a ΔG of +10.8 Kcal mol^{-1}. This energetic barrier is thought to be overcome by metabolic coupling to PPi hydrolysis and APS phosphorylation, reactions with favorable ΔG values (1). Consistent with the idea that APS kinase plays a role in facilitating ATP sulfurylase the APS kinase from *Penicillium chrysogenum* (2) and *Escherichia coli* (3) shows a high affinity for APS ($K_{m[APS]}$ = 0.25 to 1.4 μM). The enzyme from these organisms has the unusual property of being inhibited by substrate concentrations slightly above the $K_{m[APS]}$ (2,3). The explanation for this property is that the enzyme displays an ordered mechanism with ATP binding before APS. Inhibition results from the formation of a dead-end enzyme-ADP-APS or an enzyme-APS complex. A bi-functional ATP sulfurylase/APS kinase exists in animals (4,5) that may carry out substrate channeling (5). Plants are like fungi and bacteria in that they have separate enzymes (6,7).

In plants PAPS is used as a sulfuryl donor for a number of different sulfotransferases (8). It is not thought to be the substrate for sulfate reduction leading to cysteine formation, rather, APS serves this function (8). In contrast, microorganisms use PAPS as a direct intermediate in the cysteine biosynthetic pathway (9,10). Due to the limited function of APS kinase in plants it was of interest to determine whether it differs kinetically from that in *E. coli* and *P. chrysogenum*. We cloned a cDNA encoding APS kinase (*APK*) from the higher plant *Arabidopsis thaliana* by functional complementation of a *met14* (APS kinase) mutant strain of *Saccharomyces cerevisiae* (6) and later found that *APK* can also complement an *E. coli cysC* (APS kinase) strain (7). Here we report on the cloning of the *APK* gene and on the properties of the recombinant enzyme.

MATERIALS AND METHODS

Bacteriological media were prepared as described in Miller (11). Sambrook et al. (12) was followed for nucleic acid protocols. The Bradford assay (13) was used for measurement of protein concentration.

Genomic DNA isolation, RNA isolation and blotting was carried out as described in (12). The DNA probes in all nucleic acid hybridization experiments were labelled with the random primer method (14) using [α-^{32}P]dCTP (3000 Ci mmole^{-1}).

A. thaliana plants for mRNA expression studies were grown hydroponically in a nutrient medium optimized for Crucifers (CNM). CNM contains 1.6 mM $Ca(NO_3)_2 \cdot 4H_2O$, 0.4 mM $MgSO_4 \cdot 7H_2O$, 1 mM

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KNO_3, 0.2 mM KH_2PO_4, 50 μM $FeCl_2 \cdot 4H_2O$, 50 μM Na_2EDTA, 11 μM H_3BO_4, 2.5 μM $MnCl_2 \cdot 4H2O$, 0.15 μM $H_2MoO_4 \cdot 2H2O$, 0.1 μM $CuCl_2$, 0.2 μM $ZnCl_2$ and was adjusted to pH 6.7 with HCl. Plants were grown for 45 days at 23°C with a 12 hours light/12 hours dark cycle under 4 × 40 watt fluorescent bulbs and CNM was exchanged every 5 days. For sulfate starvation CNM was modified by replacing $MgSO_4 \cdot 7H_2O$ with 0.4 mM $Mg(NO_3)_2$ and KNO_3 was reduced to 0.2 mM in order to maintain total NO_3 at 4.2 mM. The plants were transferred to this solution and they were incubated for the specified time prior to analysis. The DNA probes used for RNA blotting included the *APK* cDNA (6), the *APR1* cDNA (U43412) encoding *A. thaliana* APS reductase (15), and the *A. thaliana* expressed sequence tag clone 142F20T7 (GenBank accession T76088) encoding the *AST68* sulfate transporter (16).

The genomic DNA encoding *APK* was cloned from an *A. thaliana* genomic library (17) (Arabidopsis Biological Resource Center, Ohio State University) following the plaque lift procedure and using the *APK* cDNA as a probe. The gene was sequenced on both strands with an Applied Biosystems, Inc., 373 DNA sequencer.

The transcriptional start site was determined by primer extension analysis (12) with an oligonucleotide (5′-AGAAGCCATCGAAAG-CCCTAATAGC-3′) complementary to a region near the translational start codon of *APK*. RNA from *A. thaliana* leaves (1 μg) and primer (0.5 μg) were hybridized in formamide buffer at 55°C. Reverse transcription was carried out with 20 μCi of [α-^{32}P]dCTP (3000 Ci mmole^{-1}) (final 0.28 μM) and 2 units of M-MLV reverse transcriptase (Promega, Inc.). The reaction was incubated for 10 min at 37°C. A Sanger sequencing reaction was carried out with the same primer and the cloned genomic *APK* clone using Sequenase 2.0 (US Biochemical). The extension product and sequencing reaction were analyzed by denaturing gel electrophoresis and autoradiography.

An organelle import assay was used to determine if the *APK* protein is chloroplast localized. The *APK* cDNA, cloned into the *EcoRI* site of pBluescriptSK+, was linearized with *PvuII*, transcribed with T3 RNA polymerase, and then translated in a rabbit reticulocyte extract (Promega) using [3,4,5-^{3}H]leucine (168 Ci mmole^{-1}). The import assay was carried out with pea chloroplasts (18).

APK was expressed as a recombinant protein by cloning the cDNA as a 980 bp *BglII-BamHI* fragment into the *BamHI* site of pGEX-2T (Pharmacia) and separately, into pMAL-c2 (New England Biolabs). Both constructs were used for protein expression and purification according to the manufacturers protocol. The protein preparations were typically more than 95% pure based on analysis by SDS-PAGE and staining with coomassie blue. The expression plasmids were confirmed to produce active APS kinase using a complementation assay. Both plasmids were able to functionally complement the cysteine auxotrophy of the APS kinase mutant of *E. coli*, strain JM81A [*cysC92, tfr-8*] (*E. coli* Genetic Stock Center, Yale University) on M-9 medium lacking Cys and Met and containing 0.1 mM IPTG (7).

APS kinase activity was determined with a continuous spectrophotometric assay that couples ADP production to NADH oxidation (2). The unit of activity is defined as μmol min^{-1}. The original procedure was modified by reducing the reaction volume to 300 μL. Absorbance at 340 nm was monitored over 5 minutes at 25°C. An important feature of the assay is that APS and ATP are regenerated (with P1 nuclease and pyruvate kinase) maintaining a constant level of these APS kinase substrates. The recombinant enzyme was tested for APS sulfotransferase activity exactly as described (19) with up to 100 μg of protein added to the reaction.

RESULTS AND DISCUSSION

Cloning and Analysis of the APK Gene

Southern blot analysis of *A. thaliana* with the *APK* cDNA showed that there is only 1 hybridizing DNA fragment under low stringency washing conditions suggesting that there is only a single locus with significant homology to *APK* (not shown). A genomic DNA library was screened and two individual clones isolated that were found to be overlapping fragments of the same genomic DNA region. The region hybridizing with the *APK* cDNA was localized to a 12.6 kbp *SstI-SmaI* fragment and a portion of this fragment was sequenced revealing the entire *APK* coding sequence and the 5′ and 3′ regions. The genomic map and position of the *APK* gene are shown in Fig. 1. The coding sequence is identical to the 1077 bp *APK* cDNA but it contains 543 bp of additional nucleotide sequence comprising 6 introns. The coding exons form an 828 bp open reading frame (ORF) with 276 codons predicted to encode a 29,786 Da protein. The intron-exon structure of the *APK* gene is completely different from the interrupted APS kinase genes from *P. chrysogenum* and *Emericella nidulans* (GenBank Accession U39393 and Y08866).

The translational initiator codon of the *APK* gene is not possible to predict with certainty because there are 4 in-frame methionine codons within the first 41 codons and none of them fits well with the consensus for translation initiation sites of plant genes (AACAATGG) (20). An indication that translation may begin at one of the first methionine codons is that the sequence of the resulting amino terminal 40 amino acids resembles a transit peptide for transport into plastids.

To identify the promoter region of *APK* the transcriptional initiation site was mapped by primer extension analysis. Total RNA isolated from *A. thaliana* leaves showed a single initiation site located 120 bp upstream of the putative translation initiator codon (not shown). Several potential plant promoter sequences are evident immediately 5′ of the transcription start site.

Import of the APK Polypeptide into Chloroplasts

The localization of the *APK* protein was studied using an in vitro chloroplast import assay. In vitro expression gave two products of ~28 and ~30 kDa (Fig. 2, lane 1). The larger product is more abundantly synthesized. This may result from translational initiation at more than one of the 4 possible initiation codons. After incubation with isolated chloroplasts in a reaction lacking ATP there is no change in the APK translation products (lane 2), however after incubation with chloroplasts in a reaction containing ATP a polypeptide of 26 kD is formed (lane 3). Protein import into chloroplasts is known to be ATP-dependent and is associated with proteolytic processing into a mature form that is smaller than the precursor protein (21). To determine whether the 26 kDa product is imported rather than being associated with the chloroplast surface the chloroplasts from an import reaction where re-purified and then treated with thermolysine, a protease that is unable to penetrate the chloroplast envelope. Lane 4

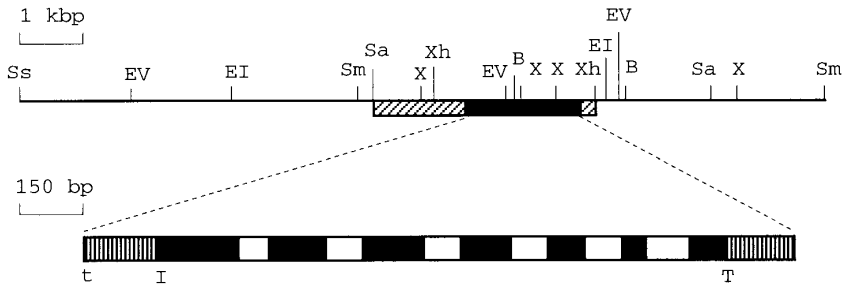

FIG. 1. Restriction map of a 12.6 kbp *A. thaliana* genomic fragment containing the *APK* gene and the structure of the *APK* gene. The upper line shows a restriction map of the *APK* genomic region. The box shows the sequenced region found in GenBank accession U59759. The solid box is the portion of the gene that is expressed and is depicted at a larger scale in the lower part of the diagram. In the lower diagram the coding exons are indicated by solid boxes and the hatched boxes show the 5′ and 3′ non coding regions. The introns are indicated by open boxes. t indicates the transcriptional start site; I and T indicate the translational initiation and termination codons. A scale bar is indicated to the upper left of each diagram. Restriction sites are as follows: Ss, *SsTI;* EV, *EcoRV;* EI, *EcoRI;* Sm, *SmaI;* Sa, *SalI;* X, *XbaI;* Xh, *XhoI;* B, *BamHI.*

shows that only the 26 kDa product is associated with the chloroplasts and that it is resistant to thermolysine. These results are consistent with the idea that the 26 kDa polypeptide is able to localize within chloroplasts and suggests that the enzyme encoded by the *APK* gene could be localized within chloroplasts in plant cells.

Expression of APK mRNA

Blot experiments were carried out to determine whether the steady state level of *APK* mRNA is influenced by plant growth conditions. Sulfate starvation has been shown to induce the accumulation of mRNA for sulfate permease (16,22) and APS reductase (16), two other proteins involved in sulfate assimilation in plants. *A. thaliana* plants grown hydroponically under autotrophic conditions for 45 days were exposed to sulfate-free nutrient medium for up to 4 days. The results of RNA blotting showed that *APK* mRNA is unaffected by this treatment in either roots or leaves. In the same samples the level of APS reductase and sulfate permease mRNA was significantly increased in roots. The results of RNA blot analysis of roots is shown in Figure 3 the results of the leaf RNA blot are not shown. After sulfate starvation there is a steady increase in *APR1* and *AST68* mRNA over 96 hours, but no increase in *APK* mRNA over the same time period. The blotting experiment also revealed that *APK* mRNA is nearly as abundant in roots as it is in leaves of *A. thaliana* suggesting that the enzyme may be expressed in both photosynthetic and non-photosynthetic plant tissues.

Kinetic Analysis of the Recombinant APK Enzyme

Recombinant *APK* was synthesized as a fusion protein with glutathione-*S*-transferase (GST-APK) or as a

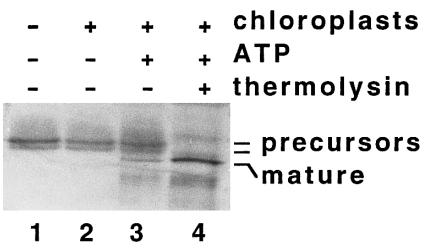

FIG. 2. Import of *APK* polypeptide into isolated chloroplasts. *APK* polypeptide was synthesized *in vitro* in a rabbit reticulocyte extract with [^{3}H]leucine. Radioactively labeled protein was analyzed by electrophoresis on a denaturing 10% (w/v) polyacrylamide gel followed by autoradiography. The experimental parameters are indicated above the photograph. The position of the precursor polypeptides (~30 and ~28 kDa) and the mature polypeptide (~26 kDa) is indicated to the right of the photograph. The translation products, lane 1; the translation products incubated with chloroplasts in a reaction lacking ATP, lane 2; the translation products incubated with chloroplasts in a reaction containing 10 mM Mg-ATP, lane 3; a reaction with chloroplasts and Mg-ATP followed by re-isolation of intact chloroplasts and then treatment with thermolysin, lane 4.

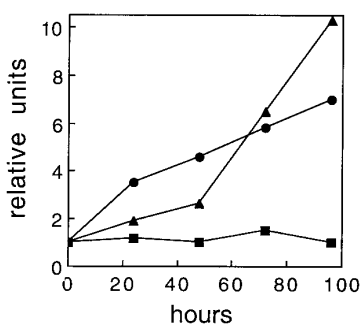

FIG. 3. RNA blot analysis. *A. thaliana* plants were transferred to sulfate starvation medium. Twelve plants were harvested at various times after sulfate starvation and pooled together for RNA was extraction. mRNA for *APK* (squares), *APR1* encoding APS reductase (circles), or *AST68* encoding sulfate permease (triangles) was measured by blotting. Ten micrograms of total RNA from each sample was electrophoresed on a 0.8% (w/v) agarose formaldehyde gel. After hybridization with the appropriate DNA probe the relative signal intensities were measured using a Molecular Dynamics, Inc., Phosphorimager.

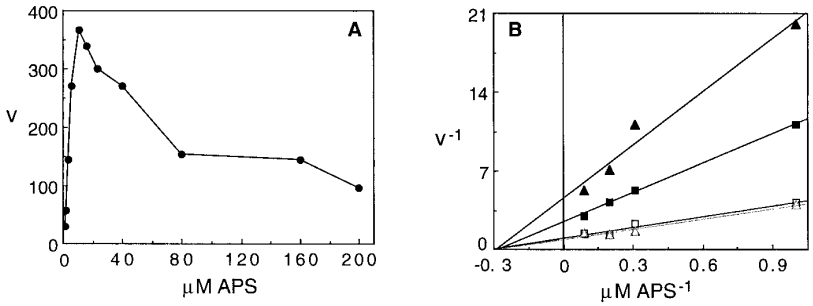

FIG. 4. Kinetic analysis of MAL-APK. A, reaction velocity (nmol NAD+ formed min^{-1}) at different fixed concentrations of APS measured with 5 mM ATP. B, reciprocal plot of velocity measurements at different sub-inhibitory APS concentrations carried out at different fixed concentrations of ATP: 0.5 mM (closed triangle), 2 mM (closed square), 5 mM (open triangle), 10 mM (open square). The kinetic constants were calculated by least-squares non-linear regression analysis (25).

fusion with the maltose binding protein (MAL-APK). All attempts to isolate APS kinase from the fusion protein by digestion with thrombin or factor Xa (the proteases recommended by the supplier of the expression vectors) resulted in degradation of APS kinase. This occurred even though there are no thrombin or factor Xa recognition sites in the *APK* protein sequence. We suspect that plant APS kinase may be very sensitive to degradation by contaminating proteases in the recombinant protein preparation or in the commercial thrombin and factor Xa preparations. Interestingly, the purified *APK* fusion protein is very stable with no loss of catalytic activity over a period of 1 year in storage at −70°C or after incubation for 24 hr at room temp.

Since the fusion proteins were found to be functional the kinetic experiments were carried out with the fusion proteins. A coupled assay was used to measure APS kinase activity. Titration of APS into reaction mixture containing 5 mM ATP showed that the velocity increases linearly up to 10 μM APS but is inhibited at higher APS concentrations (Fig. 4A). In this experiment the maximal velocity is 0.3 units mg^{-1} protein. The same results were obtained with 4 different enzyme preparations (two MAL-APK and two GST-APK) although the specific activities ranged from 0.3 to 0.5 units mg^{-1} protein. The reaction rates measured at sub-inhibitory levels of APS at varying fixed levels of ATP showed lines that intersect on the $[APS]^{-1}$ axis when displayed on a reciprocal plot (Fig. 4B). This analysis shows that the theoretical V_{max} is 1.2 units mg^{-1} protein and the kinetic constants (K_m) are 3.6 μM for APS and 1.9 mM for ATP (determined from the V^{-1} axis intercept values in Fig. 4B). All the preparations showed the same kinetic constants. The difference between the maximal observed velocity and the theoretical V_{max} is likely due to enzyme inhibition observed at high APS concentrations. These results are similar to those reported for *P. chrysogenum* (2) and *E. coli* (3) where it was interpreted to mean that APS is a noncompetitive inhibitor in relation to ATP. It should be noted that since the activity measurements were conducted with the APK fusion proteins of which ~65% is either maltose binding protein (MAL-APK) or glutathione-*S*-transferase (GST-APK), a more realistic specific activity could be ~3 fold greater, i.e. 0.9 to 1.5 units mg^{-1}. This value is well below the specific activity of APS kinase from *P. chrysogenum* (24 units mg^{-1}) and *E. coli* (153 units mg^{-1}), but it is close to the specific activity reported for pure APS kinase from *Chlamydomonas reinhardii* (2.2 units mg^{-1}) (23) and *S. cerevisiae* (0.8 units mg^{-1}) (24).

A recent report described that APS kinase from *A. thaliana* is able to catalyze the reduction of APS to sulfite under in vitro conditions in the presence of DTT (19). This reaction is similar to that reported for the sulfate assimilation enzyme in plants termed APS sulfotransferase (APS reductase). The result was interpreted to mean that APS sulfotransferase activity is an artifact resulting from an unphysiological side reaction of APS kinase (19). We tested purified recombinant APS kinase fusion proteins for this activity but none was observed. There are several reasons why APS sulfotransferase activity may not have been observed. The initial observation in reference (19) was not carried out with pure APS kinase and it could be that the activity resulted from an interacting component in the cell extract; that pure plant APS kinase does not display APS sulfotransferase activity. Another possibility is that the fusion partner in the *APK* recombinant proteins inhibits the APS sulfotransferase activity of APS kinase.

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