

Molecular and Catalytic Properties of *Arabidopsis thaliana* Adenylyl Sulfate (APS)-Kinase¹

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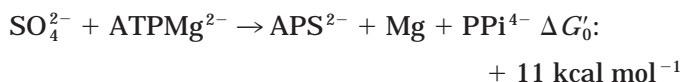
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A cDNA clone (*Atakn1*) from *Arabidopsis thaliana* encoding APS-kinase (EC 2.7.1.25) was investigated for structural and catalytic properties of the gene product. Recombinant his₁₀-*AtAkn1* formed PAPS at a V_{\max} of 7.35 U mg⁻¹. The K_m for APS was 0.14 μM and for ATP 147 μM. APS caused a severe substrate inhibition (K_i 4.5 μM). The type of inhibition is uncompetitive with respect to MgATP. High ionic strength and reducing thiols stabilized the enzyme activity. Plant APS-kinase is regulated *in vitro* by the redox charge with thioredoxin as essential activator. Mutagenesis of a serine in S182C and S182F presumed to be involved in the transfer of the phosphoryl group had no effect upon catalytic activity. Using a yeast two-hybrid system with *AtAkn1* as bait, an interacting clone was detected from a cDNA library of *A. thaliana* cv. Columbia that codes for an APS-kinase iso-form (*Atakn2*). Complementation of APS-kinase-deficient *Saccharomyces cerevisiae met14* showed that *AtAkn2* is functionally active as APS-kinase. It was immunologically related to *AtAkn1* and presumably represents a plastidal iso-form of the plant APS-kinase gene family. © 2001 Academic Press

Key Words: APS-kinase; ATP:adenylylsulfate 3'-phosphotransferase; *Arabidopsis thaliana*; APS; adenylylsulfate; PAPS; phosphoadenylylsulfate; sulfate activation; sulfate assimilation; thioredoxin.

ATP-sulfurylase (MgATP:sulfate adenylyltransferase, EC 2.7.7.4) and APS-kinase (MgATP:adenylylsulfate 3'-phosphotransferase, EC 2.7.1.25) catalyze the initial steps in sulfate activation. Both enzymes are energetically coupled:



and—including the hydrolysis of pyrophosphate by inorganic pyrophosphatase ($\Delta G'_0$: -6 kcal mol⁻¹)—convert inorganic sulfate into the highly reactive sulfonyl compound PAPS.⁵ APS-kinases are small phosphotransferases (22–27 kDa) that transfer the phosphate group from ATP to the 3'-OH group of the ribose moiety in adenylylsulfate (APS). All APS-kinases that have been studied *in vitro* exhibited an extremely high affinity for APS with a K_m in the range of 10⁻⁶ M. Another characteristic property of APS-kinase is its potent substrate inhibition that is seen in the absence of ATP. The reaction mechanism of APS-kinase has been studied extensively from *Penicillium chrysogenum* and from *Escherichia coli*. It has been a matter of considerable debate in the past but with the high-resolution structure of APS-kinase from *P. chrysogenum* (1) at hand, catalytic constants and groups involved in substrate binding and turnover can now be refined on a rational structural basis.

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⁵ Abbreviations used: akn, APS-kinase; APS, adenylylsulfate; DTT, dithiothreitol; NBT/BCIP, nitroblue tetrazolium/5-Br-4-Cl-3-indolylphosphate; NPCF-agarose, 4-nitrophenylchloroformate agarose; PAPS, phosphoadenylylsulfate; trx, thioredoxin.

Plant APS-kinases have been studied in only a few cases (2–4) and although APS-kinase activity has been found in leaf tissue or plastids from several higher plants, kinetic studies of a purified enzyme were only reported from a green alga (4). The *Chlamydomonas reinhardtii* enzyme was as efficient as its counterparts from fungi or bacteria; in addition to the former enzymes, it appeared regulated by reduced thioredoxin (5). The role APS-kinase plays in higher plants is not well understood. PAPS is accumulated in large amounts in cell extracts (6) but the current view is that plants use APS for cysteine biosynthesis in the plastid while PAPS is used in the formation of sulfate esters (7). However, sulfate esters like sulfated flavonols, glucosinolates, plant steroids, and phytosulfokines (8) are synthesized in the cytoplasm and localized in the vacuole and Golgi system. The biosynthesis of the sulfolipid (sulfoquinovosyldiacylglyceride) also depends on APS as sulfonyl group donor *in vitro* (9) so that the function of a plastidal APS-kinase is unclear. Steady-state concentrations of APS in the plastid are not known, but when estimated from *in vitro* assays with purified enzymes that synthesize, bind, and metabolize APS, the concentration of free APS in the plastid may be expected in the nanomolar range (11, 12). If active under such conditions in the same compartment, APS-kinase would accumulate PAPS and deprive other APS consuming reactions of its substrate. Detailed investigations of the reaction mechanism and regulation of APS-kinase are not available from plants but from *P. chrysogenum* (12) and *E. coli* (13, 14). The fungal enzyme seems to follow a compulsory ordered mechanism. Steady-state kinetics and equilibrium binding studies suggested that APS can occupy the sulfonucleotide binding site after PAPS has left the enzyme but before the ExMgADP complex dissociates (15, 16). This type of mechanism differs from the mechanism reported for the APS-kinase from *E. coli* where binding of APS before ATP resulted in an enzyme-APS dead-end complex. Binding of ATP before APS was shown to give an enzyme-phosphate intermediate that could transfer the phosphate group to APS (14).

Of the four *Arabidopsis thaliana* APS-kinase genes that are recorded in the GenBank-EMBL database (release 118.0), two are predicted to be located in the plastid (i.e., *akn1* and *akn2* (17, 18)) whereas the two putative APS-kinases detected in the course of genome sequencing projects are not yet characterized but may represent cytoplasmic forms. In the present paper, we investigated structural and catalytic properties of recombinant APS-kinase (Akn1) from *A. thaliana*. In order to elucidate possible protein-protein interactions between ATP-sulfurylase and APS-kinase we employed a yeast two-hybrid system using APS-kinase as bait. The new APS-kinase clone was tested for functional complementation of the yeast *MET14* mutant.

To find a reliable and sensitive method to determine the catalytic properties and kinetic constants of the recombinant APS-kinase in the presence of submicromolar concentrations of substrates, a new assay is introduced using recombinant PAPS-reductase as enzymatic probe to test the amount of reaction product PAPS. Steady-state measurements of the recombinant his₁₀-APS-kinase are made to describe the pattern of substrate inhibition from a plant APS-kinase. The possible role of serine182 as phosphoryl group acceptor was investigated by site-specific mutagenesis.

MATERIALS AND METHODS

Strains of bacteria, yeast, and plasmids. Strains used were as follows. *E. coli* XL1 Blue MRF': $\Delta(mcrA)183$, $\Delta(mcrCB-hsdSMR-mrr)173$, *endA1*, *supE44*, *thi-1*, *recA1*, *gyrA96*, *relA1*, *lac*⁻, [F', *proAB*, *lacI*^q Δ M15, Tn10(Tet^r)] (Stratagene, La Jolla, CA); BL21(DE3):F', *ompT*, *hsdS_B* (r_Br_S), *gal*, *dcm* (DE3) (Novagen); TG1: *supE*, *thi*, *hsd Δ 5, $\Delta(lac-proAB)$, F'[*traD36*, *proAB*, *lacI*^q, *lac Δ M15]. *Saccharomyces cerevisiae* CC366-6B (MET14): *mat α* , *his3*, *ura3*, *met14* (Y. Surdin-Kerjan, CNRS, Gif-sur-Yvette); D237-10b:*mat α* (ATCC24657); L₄₀: *mat α* , *his3*, D200*trp1*-3112, *ade2*, LYS2: ::(4*lexAop* - HIS3) 3:::(8*lexAop-lacZ*)GAL4 (Invitrogen, Groningen). The following phagemids, cloning, expression, and shuttle vectors were used: pBluescriptII KS/SK (Stratagene), pET16b (Novagen, Madison, WI), pYPGEM14, pYPGE15, pHybLex/Zeo, pYESTrp (Invitrogen), pGAD10 (Clontech, Palo Alto, CA). The *Matchmaker* cDNA library from *A. thaliana* was purchased from Clontech.**

Growth of bacteria and expression of enzymes. *E. coli* BL21(DE3) transformed with pET derivatives was grown under vigorous aeration in a chemostat at 32°C in LB medium, 100 μ g ml⁻¹ ampicillin, and glycerol (1% v/v) at pH 7.5 (19). His fusion proteins were purified on Talon (Clontech) metal-affinity resin under nondenaturing conditions and stored in 50% (v/v) glycerol at -20°C. The average yield per liter of broth was 14 mg of his₁₀-AtAkn1.

Synthesis of [³⁵S]-nucleotides. [³⁵S]PAPS was prepared enzymatically from [³⁵S]SO₄²⁻ (Amersham-Pharmacia, Braunschweig) and ATP using recombinant his₁₀-APS-kinase from *A. thaliana* (AtAkn1) in a coupled system containing ATP-sulfurylase, inorganic pyrophosphatase (Sigma, Deisenhofen), pyruvate-kinase and phosphoenolpyruvate (Roche, Mannheim). [³⁵S]APS was produced enzymatically from [³⁵S]PAPS using P1 nuclease (Roche) and purified by ion-exchange chromatography on DEAE 650 resin (Merck, Darmstadt) as described previously (20). [³⁵S]Nucleotides were assayed by reversed-phase paired-ion HPLC as described earlier (21).

Enzymatic assays. Recombinant his₁₀-APS-kinase from stocks stored at -28°C in 50% glycerol at 0.2 mg ml⁻¹ was preincubated for 4 min at 25°C in 10 mM DTT plus 2 μ M thioredoxin 1 in 0.4 M Tris-HCl, pH 7.4, in order to restore full activity. In steady-state analysis the experiments were repeated five times using three independently prepared his₁₀-APS-kinase proteins. Data points were collected and averaged from *n* \geq 10 individual measurements. Curves obtained from these data were fitted by nonlinear regression to the velocity equation in Ref. (22) describing an ordered bireactant sequence with a dead-end complex.

Product determination by HPLC. Ten to 100 ng his₁₀-APS-kinase was assayed in 50 mM Tris-HCl, pH 7.4, 1 mM MgCl₂, 200 mM NaCl, 2 mM DTT, 0.1 μ M thioredoxin, and 5 to 200 μ M ATP in a volume of 0.1 ml. The enzyme was preincubated in this mixture for 30 min before the reaction was started with 0.5 to 5 μ M [³⁵S]APS. The reaction was stopped by injection of 50 μ l of 1 mM N-Br-succinimide in acetonitrile and samples of 25 μ l were analysed by HPLC (21).

Product determination by PAPS-reductase. [³⁵S]PAPS formed in the APS-kinase reaction is converted in a second incubation to [³⁵S]sulfite by recombinant PAPS-reductase with reduced thioredoxin as reductant. PAPS formation was linear (3.72 U mg⁻¹) when 1–5 ng of APS-kinase was allowed to react for 3 min before addition of EDTA (67 mM final concentration) terminated the reaction. An aliquot of 50 μl of the PAPS-containing sample was withdrawn and added to 500 ng recombinant his₁₀-PAPS-reductase (1–2 U) as indicator enzyme in 50 mM Tris-HCl, pH 8.0, 5 mM DTT and 10 μM thioredoxin at a final volume of 0.15 ml. The PAPS-reductase was allowed to react for 30 min at 25°C before [³⁵S]sulfite is determined as in Ref. (23).

Protein determination and immunoblotting. The concentration of protein was determined colorimetrically (24) or by absorbance at 260/280 nm. SDS-PAGE was run on 12.5% gels replacing β-mercaptoethanol with 10 mM DTT. Polyclonal anti-APS-kinase antibodies from rabbit were purified by immunoaffinity using his₁₀-AtAkn1 as ligand coupled to NPCF-agarose (Devitron, Castrop-Rauxel). Cell extracts that were separated by SDS-PAGE were transferred to nitrocellulose according to Michov (German Patent No. 4127546 cited in 25). Immunoprecipitates on nitrocellulose Protran BA79 (Schleicher-Schuell, Dassel) were visualized using phosphatase-conjugated goat anti-rabbit antibodies and NBT/BCIP for staining. The limit of detection in a Western blot was ~16 μg L⁻¹ of AtAkn1 using affinity-purified anti-akn IgG diluted 1:10000.

Molecular methods. Basic cloning methods were carried out according to Sambrook *et al.* (19). *E. coli* was transformed according to Hanahan (26) or by electroporation when plasmids were used that were isolated from yeast. Rapid small-scale transformation of yeast was according to Rose (27). Transformation with libraries was done according to Ref. (31). For DNA sequencing the AutoRead sequencing kit (Amersham-Pharmacia) was used in an automated system.

Mutagenesis. Primers for the mutagenesis of S182 by overlap extension (32) were pAKCS182wob5', 5'-NCATTGCGAGTTT-GATATNTCCTTATAGAA, and pAKSC182wob3', 5'-NAAGCGTC-CCTATCTGTTCTATAAGGAGATA, substituting N with A, G, C, and T. The primers Ak-h and pET-APSK5', 5'-CTTGTGTTCATAT-GGATGGATCTCAAACCTC, were used as second (outer) set of primers. The PCR products were cloned via restriction sites (underlined).

Two-hybrid system. pHLZakn1 serving as bait was constructed by cloning of a 711-bp EcoRI/XhoI PCR fragment into pHybLex-Zeo. pATG1/1 containing *Atakn1* was used as DNA template. The plant Matchmaker cDNA library (Clontech) was cloned in pGAD10. DNA (60 μg) of this library was used to transform *S. cerevisiae* L₄₀ harboring pHLZakn1. Transformants were plated on minimal medium (-his, -leu, +ade, +trp) in the presence of zeocin/amino-triazol. Twenty-four putative interaction clones per 0.852 × 10⁶ transformants were isolated and submitted to a second round of screening. The plasmids were extracted from the yeast colonies and amplified in *E. coli* TG1 selecting for amp^r. Cotransformation of *S. cerevisiae* L₄₀ harboring pHLZakn1 produced three positive clones that grew on selective his⁻ medium where pHLZakn1 as prey recovered pGADakn2 as bait.

RESULTS

A. thaliana seems to contain four independent copies of genes that encode for APS-kinases. At least two of these cDNAs (termed *akn1* and *akn2*, Fig. 1) encode for functionally active enzymes. *AtAkn1* has been used previously to complement a *cysC* mutant from *E. coli* (17) whereas *Atakn2* is used here to complement the yeast APS-kinase mutant *met14*. The polypeptide sequence encoded by *akn2* consists of 293 amino acids (*M_r* 31,977 Da) and is 75.6% identical with *AtAkn1*.

Recombinant *AtAkn2* cross-reacted with monospecific antibodies that were obtained from antisera raised and purified against recombinant *AtAkn1* (Fig. 2). When compared with APS-kinase from *E. coli* or the putative gene product of *akn3* the N-terminus appears extended by a large transit peptide consisting of 86 residues. A protease site is predicted at position 59–60 (URL: <http://www.cbs.dtu.dk/services/ChloroP>) and if digested accordingly *Akn2* would yield a mature product of 25.8 kDa. Processing and import into the plastid are not yet investigated but predicted (URL: <http://www.inra.fr/Internet/Produits/Predotar/>). Presumably *Atakn2* represents a plastidal iso-form of APS-kinase with its gene located on chromosome IV while *AtAkn1* is on chromosome II. Primarily, isolation of the *AtAkn2* encoded iso-form showed that APS-kinase in plants is not a single copy gene as suggested previously (4) but a member of the small gene family of APS-kinases. Inspection of the genome (30) revealed two highly related DNA regions that may encode two further APS-kinase iso-forms: a small APS-kinase of 23.1 kDa and a large protein of 32.2 kDa. The gene encoding for the small iso-form is located on chromosome III and may represent a cytoplasmic protein while the large iso-form found on chromosome V resembles a preprotein with the ER, periplasm, or vacuolar membrane system as target.

AtAkn1 and *AtAkn2* are freely diffusible proteins. Protein-protein interactions that would have been expected if plant APS-kinase(s) were constituents of protein complexes as in mammals were not observed. However, as the cDNA of *AtAkn2* was originally isolated from a Matchmaker library presenting *akn1* as bait (18) both proteins must have interacted presumably by forming a heterodimer with *AtAkn2*. Evidence for a dimerization between APS-kinase monomers was also obtained in the Hybrid Hunter system with *CrAkn* from *Catharanthus roseus* and *A. thaliana* when *AtAkn1* served as bait and *CrAkn* as prey.

Catalytic properties of the plant enzyme were investigated with recombinant *AtAkn1*. We introduced a new assay system for the measurement of APS-kinase activity by combining the kinase reaction with the reaction of PAPS-reductase. In the PAPS-reductase coupled assay [³⁵S]APS is first converted to [³⁵S]PAPS in the APS-kinase assay and [³⁵S]PAPS is converted to [³⁵S]SO₃ by PAPS-reductase added to the same sample. Sulfite is detectable even against a high background of ³⁵S-labeled APS or PAPS. With excess EDTA APS-kinase is completely inhibited and PAPS formed in the first reaction is quantitatively converted to sulfite in the second. PAPS formation was linear when 1–5 ng of his₁₀-APS-kinase was employed. (Within the temperature interval from 15 to 35°C *Q*₁₀ equals 1.94 when the rate is monitored from pH 6.8 to 8.4, data not shown.) *AtAkn1* was rapidly inactivated by dialysis, dilution, or

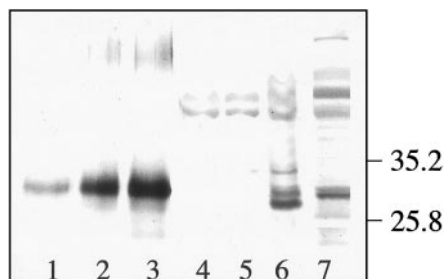


FIG. 2. Expression of recombinant plant APS-kinase *akn1* and *akn2*. Western immunoblotting of crude extracts from host bacteria after SDS-PAGE and electrotransfer containing: (lane 6) *AtAkn1-his₆* in pKK223 using JM105 as host (25 μ g protein cell extract); (lane 7) *AtAkn2-his₆* in pBluescriptKSII+ and XL1-Blue (25 μ g); lanes 1–3 contained 0.4, 0.8, and 1.2 ng of purified *his₁₀-AtAkn1* as reference; controls: (lane 4) JM105 and (lane 5) JM105 plus vector. Immunodetection with phosphatase-conjugated AB using BCIP/NBT for staining. Dilution 1:10⁴ of first AB, rabbit-anti-*akn1*, as purified IgG; second AB, 1:5 $\times 10^3$ goat-anti-rabbit, cross-reaction with APS-kinase from *E. coli* is suppressed by incubation of the first antibody with wild-type bacterial cell extract. Position of prestained carbonic anhydrase (35.2 kDa) and lactoglobulin (25.8 kDa) as indicated.

mized conditions a reaction rate of 3.9 μ mol PAPS $\text{mg}^{-1} \text{min}^{-1}$ (=100%) was obtained in the coupled assay with PAPS-reductase as auxiliary enzyme. The activation by thioredoxin was considered essential when compared to chemical reduction by DTT (39% at 5 mM DTT, 8.2% at 2 mM DTT, and 0.9% without reductants) because thioredoxin plus DTT not only displayed a shorter lag phase as expected for any thioredoxin-modulated protein but also doubled the portion of active enzyme (Fig. 4). One plausible explanation would be that a refolding of denatured recombinant APS-kinase proceeds stepwise. The higher level of activity obtained with thioredoxin 1 would then possibly reflect a step where the thioredoxin's disulfide reductase/isomerase activity is indispensable to facilitate the refolding of a structurally or kinetically trapped misfolded protein. When the activated enzyme was treated with oxidized glutathione the enzyme rate decreased immediately to the level that was observed before activation. Plant APS-kinase differs from bacterial or fungal APS-kinase by its high content of cysteinyl residues. Six cysteine residues (marked by asterisks in Fig. 1) in the four copies of *A. thaliana* APS-kinases are fully conserved and in addition, a less well conserved cysteine only present in *Akn1*, *Akn2* and positioned slightly different in *Akn4* is found in proximity of the N-terminus. We have reported earlier that the enzyme from the green alga *C. reinhardtii* was activated by thiols and deactivated by oxidants (5). The activation by thiols and the specific effect by thioredoxin on one side and the inactivation by an oxidant like GSSG on the other can be seen in agreement with earlier data observed for the enzyme from *S. cerevisiae* (20) and *C. reinhardtii* (5).

AtAkn1 was already saturated with APS in a range below 1 μ M—above this concentration the reaction was inhibited and the rates declined to a lower level depending on the concentration of ATP (Fig. 5). The mode of inhibition was tested according to MacRae and Segel (22). The authors showed that in an uncompetitive inhibition the concentration of APS at optimum velocity [APS_{opt}] is shifted toward the origin for increasing concentrations of ATP when the normalized velocity is replotted against the optimum concentration of APS at

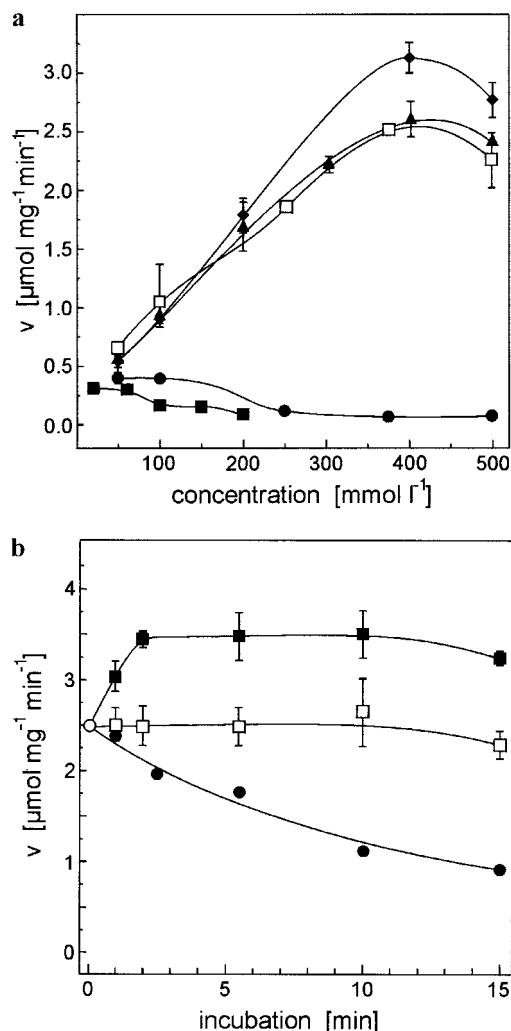


FIG. 3. (a) Activation by high ionic strength of recombinant *AtAkn1*. Inactive recombinant *his₁₀-APS-kinase 1* as isolated and stored in 50% glycerol at -20°C is reactivated by high ionic strength. The rates increased from a basal level of 0.35–0.52 to 2.5 U mg^{-1} in 0.4 M Tris-HCl, pH 7.4 (\blacklozenge), and 3.2 U mg^{-1} in 0.4 M triethanolamine-HCl, pH 7.4 (\blacktriangle). Chloride ions (\square) added to 100 mM Tris-HCl, pH 7.4, sulfate from stocks of Na_2SO_4 (\bullet), or $(\text{NH}_4)_2\text{SO}_4$ (\blacksquare). (b) Stability of *AtAkn1* and activation by thioredoxin. Enzyme activity under conditions of high salt as in a plus treatment with DTT (\square); enzyme as before but supplemented with thioredoxin 1 showing increase of the rate by 40–42% (\blacksquare). Time course of decay of activity in absence of DTT (or GSH) as reductant(s) as indicated (\bullet).

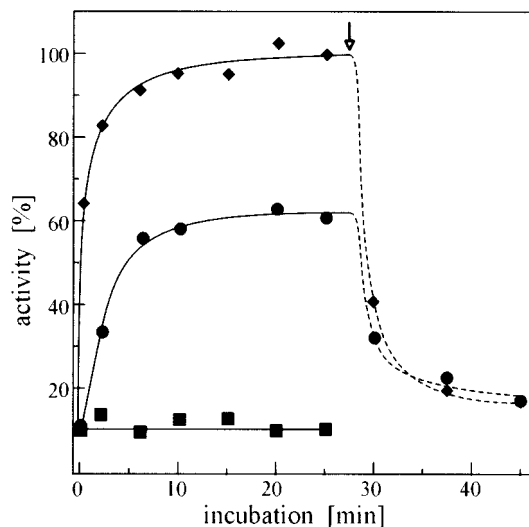


FIG. 4. Redox-control of the enzymatic activity of *AtAkn1* by thioredoxin and oxidized glutathione. APS-kinase (2.5 ng) was preincubated in a reaction mix containing ATP (1 mM) and the reductants ((\blacklozenge) *E. coli* thioredoxin 1 μM plus DTT 10 mM) for time intervals as indicated, (\bullet) DTT minus thioredoxin1, (\blacksquare) no reductants. The reaction was started by addition of APS (10 μM). PAPS formed after 180 s was quantitated by the PAPS-reductase assay. To reverse the activation, excess (=20 mM) oxidized glutathione was added after 30 min (as marked by arrow). The APS-kinase was started by addition of APS as before and allowed to react for 180 s, and 100% of relative activity corresponds to 2.7 $\mu\text{M mg}^{-1}$ of PAPS formed per test.

a constant concentration of ATP. In a competitive inhibition the velocity was predicted to increase as the MgATP is increased. When the data are treated accord-

ingly, $[\text{APS}]_{\text{opt}}$ decreased asymptotically as predicted for the uncompetitive inhibition (inset in Fig. 5). The K_m for APS was calculated from the noninhibited rates of the same set of data replotted as a double reciprocal plot of APS at different fixed concentrations of ATP (Fig. 6). The $K_{m\text{APS}}$ calculated from the x -axis intercept was 0.14 μM . V_{max} calculated from the data in Fig. 6 was 7.35 $\mu\text{mol PAPS mg}^{-1} \text{ min}^{-1}$. $K_{i\text{APS}}$ was 4.5 μM when extrapolated from an intercept replot of v_{app}^{-1} observed at zero [ATP] for different suboptimal concentrations of APS (inset in Fig. 6).

Using the coupled APS-kinase/PAPS-reductase assay we could measure initial velocities at different concentrations of ATP in a range of noninhibitory concentrations of [APS] (Fig. 7). K_m calculated from double reciprocal plots of ATP vs different fixed concentrations of APS was 147 μM . (Virtually the same K_m is obtained from the intercept replot of the data in Fig. 5 considering the noninhibited rates only.) This K_m is about 10-fold lower than the value obtained for the fungal enzymes but considerably higher than those reported for the enzyme from *E. coli* (14) and *C. reinhardtii* (3) and for the mammalian PAPS-synthetase (31). We suspect that the lower $K_{m\text{ATP}}$ values reported for the bacterial and algal enzyme may have been obscured by the inhibitory effect of APS causing a shift in the replot of v_{app}^{-1} intercepts vs $[\text{ATP}]^{-1}$.

APS-kinase from *E. coli* was reported to form a phosphorylated enzyme intermediate upon incubation with ATP (13). Serine109 was identified in *EcAkn* as an intermediate phosphoryl group acceptor. A serine lo-

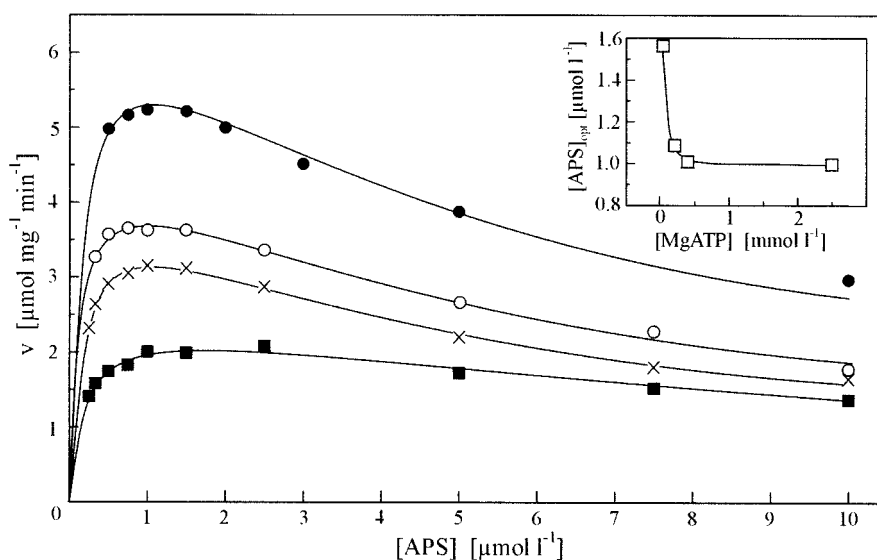


FIG. 5. Reaction velocity vs [APS] at different fixed concentrations of [MgATP]. The assay contained 400 mM Tris/HCl, pH 7.4, 1.5 mM DTT, 0.68 μM thioredoxin 1 (*E. coli*), 0.04–2.5 mM MgATP, 0.25–10 μM [^{35}S]APS, and 0.2–3 ng of purified APS kinase in 100 μl . Symbols for [MgATP]: \blacksquare , 0.04; \times , 0.22; \circ , 0.4; \bullet , 2.5 mM. Individual data points ($n \geq 10$) were collected from three APS-kinase preparations and fitted by nonlinear regression as described under Materials and Methods. Inset: $[\text{APS}]_{\text{opt}}$ vs different [MgATP] showing the decrease of $[\text{APS}]_{\text{opt}}$ at higher MgATP concentrations.

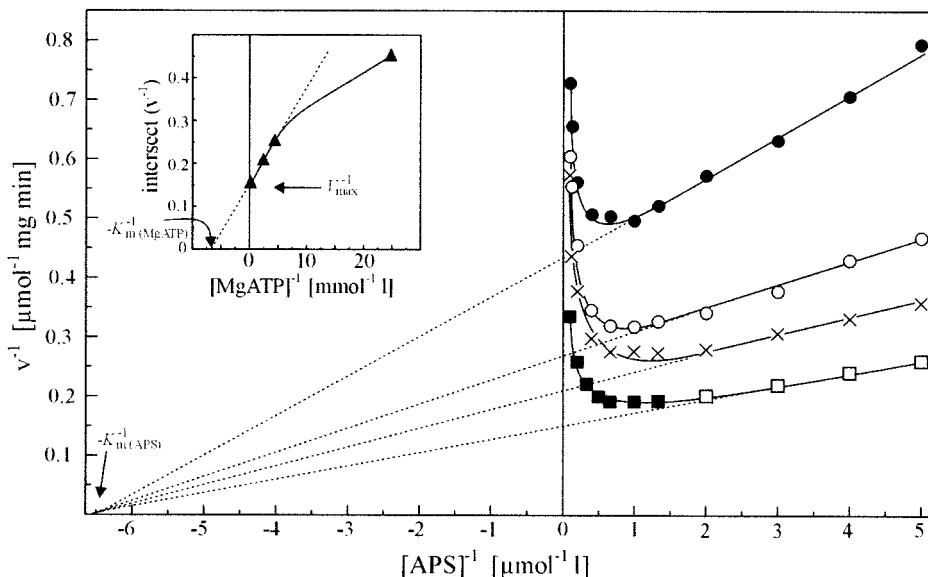


FIG. 6. Plot of reaction velocity vs [APS] at different fixed concentrations of [MgATP]. The data from Fig. 5 were redrawn in a Lineweaver-Burk diagram to demonstrate the substrate inhibition as indicated by the upward curvature of the lines. Symbols used [MgATP]: ■, 2.500; ×, 0.4; ○, 0.22; ●, 0.04 mM. The common intersect on the x axis defines the K_m for APS obtained by extrapolating the data of noninhibitory APS concentrations. Inset: Plot of intercepts (▲) with the $1/v$ axis versus $[MgATP]^{-1}$, yielding $-1/K_m$ MgATP.

cated in a corresponding position (at LIS₁₈₂P) is also found in the plant APS-kinases. In order to investigate a function in the phosphotransferase reaction as suggested for *EcAkn* we constructed two mutant proteins substituting S182C and S182F. The two mutant proteins were expressed as his₁₀-tag gene products as before and tested for activity. The catalytic activity of both APS-kinases was not affected, displaying rates that were already observed for the wild-type enzyme (data not shown). Since its mutation had no effect on the catalytic activity, we assume that serine182 in the plant APS-kinase is not required for phosphorylation of the protein. It is implied from the data that the mutant plant enzymes reacted like the enzyme from *P. chrysogenum* where mutation of the corresponding serine107 showed also no effect (12).

CONCLUSIONS

PAPS biosynthesis in *A. thaliana* is catalyzed by highly efficient APS-kinase(s). The activity of *Akn1* is submitted to a thiol-disulfide-mediated redox control. Heterologous thioredoxin (*trx1* from *E. coli*) acted as an essential activator but in view of the diversity of different plant thioredoxins it seems worthwhile to identify the homologous thioredoxin(s) that control APS-kinases in a higher plant. The plant enzyme contains six conserved cysteine residues per monomer which could be considered as regulatory target(s). Based on the 3D high-resolution structure of the *P. chrysogenum* enzyme this redox control could involve the residues cys132 and 176 which are close enough to form an

intramolecular disulfide bridge (suggested in Ref. 1). A second possibility involves cys87 and cys120 which are close enough to form an intermolecular disulfide bridge between two monomers. These residues are exposed at the surface which would allow interaction with thioredoxin. Moreover, if the plant enzyme forms a similar dimer interface, formation of a disulfide bridge could

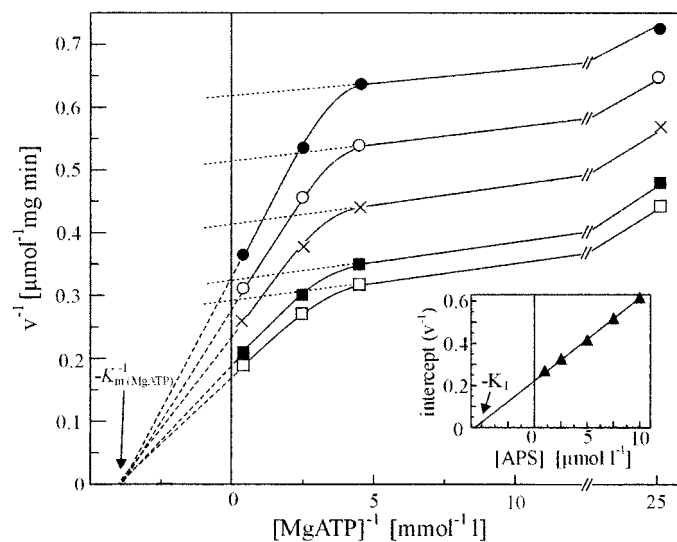


FIG. 7. Determination of K_m ATP at noninhibitory concentration of [APS]. [³⁵S]APS as substrate could be tested in the range from (□) 10, (■) 7.5, (×) 5, (○) 2.5, (●) 1 μ M at different concentrations of ATP. In the double reciprocal plot, considering the noninhibited concentrations only, a common intersect is obtained for $-1/K_m$ ATP at 147 μ M. The replot of y axis intercepts is used to determine K_i APS.

sterically affect the purine-binding site. It is interesting to note, that the N-terminal cysteine is not conserved in the putative cytoplasmic Akn3 and slightly modified in its position in Akn2 and Akn4 whereas cys132 which would be used for the intramolecular disulfide bridge is only found in Akn1 and absent from Akn2, Akn3, and Akn4.

The K_m value for APS ($K_{m\text{APS}}$ 0.14 μM) of recombinant APS-Kinase encoded by *Atakn1* was in a similar range as described for the enzyme purified from the green alga *C. reinhardtii* (3) and from *E. coli* (13, 14). Plant APS-kinase—like its bacterial and fungal counterparts—is also severely inhibited by APS. The data suggest that the inhibition by APS is uncompetitive versus ATP as it is in *P. chrysogenum* (12, 16). The K_m for ATP ($K_{m\text{ATP}}$ 147 μM), however, was higher by a factor of 20 when compared to that for *C. reinhardtii*.

Recombinant APS-kinase with a $V_{\text{max}}/K_{m\text{APS}}$ ratio of $2.8 \times 10^7 \text{ L mol}^{-1} \text{ s}^{-1}$ is a highly efficient enzyme. If APS-kinase and plant APS-reductase both reside in the plastid as freely diffusible enzymes and compete for APS under virtually identical physiological conditions, APS-kinase would presumably limit the reduction of APS because the efficiency is higher by a factor of 10^3 to 10^4 . It is not clear at present how APS-kinase and APS-reductase activity in the plastid are controlled. One may speculate, however, that the inhibition by APS of APS-kinase controls the flow of sulfate as APS-reductase activity is not affected. Yet in the plastid, the steady-state concentration of APS is not alone governed by APS-kinase or APS-reductase but also by ATP-sulfurylase which quantitatively converts APS to ATP in the presence of PPI. The concentration of pyrophosphate in leaves is 0.2–0.3 mM where 12% of the total PPI are associated with the plastid fraction (32). If freely diffusible as inorganic pyrophosphate, it would be used for ATP synthesis in the reverse reaction of ATP-sulfurylase. With its low K_m 's for APS (0.37 μM) and PPI (10 μM) and a k_{cat} of 218 s^{-1} ATP-sulfurylase would not allow accumulation of APS. In this respect, metabolic control and localization of APS-kinase and APS-reductase remain open questions which merit further work.

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