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APS-sulfotransferase activity is identical to higher plant APS-kinase (EC 2.7.1.25)

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Abstract A cDNA from *Arabidopsis thaliana* L. Heynh encoding the APS-kinase (EC 2.7.1.25) was modified by deletion of a plastidic transit peptide to enable its expression in *Escherichia coli*. The resultant protein (MW 25,761) is enzymatically active as APS-kinase and restores prototrophic growth in an APS-kinase mutant. All transformants harbouring the modified plant DNA also acquired APS-sulfotransferase activity. In the absence of ATP but provided with DTT, a tetrameric form of recombinant APS-kinase exhibits APS-sulfotransferase activity. Monospecific polyclonal antibodies raised against the APS-kinase as immunogen also reacted against APS-sulfotransferase. We propose that APS-sulfotransferase activity is a non-physiological side reaction of APS-kinase.

Key words: APS-kinase; APS-sulfotransferase activity; Gene expression; Recombinant enzyme; *Arabidopsis thaliana*

1. Introduction

The sequence of reactions that enable higher plants to assimilate inorganic sulfate differs from bacteria and lower eukaryotes. Plants were proposed to reduce adenylyl sulfate (APS) to bound sulfite rather than phosphoadenylyl sulfate (PAPS) to free sulfite:

Plants: $APS^{2-} + RSH \rightarrow AMP^{2-} + RS{:}SO_3H$
Others: $PAPS^{4-} + R(SH)_2 \rightarrow PAP^{4-} + SO_3H^- + R(S)_2 + H^+$

The activity responsible for this reaction in plants was described as APS-sulfotransferase [1,2] transferring the sulfate group from APS to an endogenous sulfite carrier. This hypothesis was supported by the identification of glutathione as carrier [3] and by in vitro studies of APS-sulfotransferases found in green algae, higher plants and *Euglena* ([4], reviewed in [5]). However, as plants were also found to contain the enzymes (APS-kinase, PAPS-reductase, sulfite reductase and *O*-acetylserine thiol lyase) and cofactors (thioredoxin, ferredoxin) that were necessary to support the reduction of PAPS via sulfite to sulfide as in bacteria or fungi ([6], reviewed in [7]), the exclusive role of the APS-sulfotransferase as suggested in [1,2] became questionable. The PAPS–sulfite reaction pathway of bacteria and lower eukaryotes depends on thioredoxin as reductant [8] which is used by PAPS-reductase to form free ionic sulfite from PAPS. In comparison to the mechanism proposed for plants, there is additional evidence for the above mechanism in bacteria and lower eukaryotes from analysis of mutants blocked in the pathway of cysteine biosynthesis [9].

One of the major obstacles in the way of solving the question of whether plants contain two independent parallel pathways for sulfate assimilation, or a plant-specific reaction sequence as proposed by Schmidt and others [1,2], is the low concentration of sulfate metabolizing enzymes in plants. As a consequence, most enzymes and reactions reported in the earlier work were studied from crude homogenates or partially enriched protein fractions – in the presence of obscuring side activities or artifacts which were not always ruled out unambiguously. If both pathways existed in the chloroplast, APS-kinase and APS-sulfotransferase would have to compete for APS as the only substrate, for which both enzymes were reported to have the same high affinity. In addition, it was observed previously that APS-kinase enriched from *Chlamydomonas reinhardii* [11] in vitro formed an intermediate sulfite when no ATP was available. Sulfite formation from APS by APS-kinase would then not be distinguishable from APS-sulfotransferase activity.

More recently, with the successful identification and cloning of structural genes encoding ATP-sulfurylase, APS-kinase, PAPS-reductase, ferredoxin-sulfite reductase, and *O*-acetylserine (thiol) lyases from higher plants and cyanobacteria (survey in [7]) access has been given to study the gene activity or expression of gene products. Yet, in this respect, over-expression of a cloned gene is also a highly attractive way to isolate and study a homogeneous active protein. When expressed in a host organism that lacks the activity in question it can be investigated under more properly defined conditions or even in the absence of obscuring side activities. In view of the disputed role of APS-kinase we isolated and characterized a cDNA from *Arabidopsis thaliana* [11] which encodes a plastidic APS-kinase. In order to express this DNA as a functional enzyme it was modified by deleting its transit peptide sequence. In this short communication we show that recombinant APS-kinase also contains APS-sulfotransferase activity. Using monospecific polyclonal antibodies raised against the APS-kinase it can be demonstrated that both activities originate from the same gene product encoded by the plant APS-kinase gene.

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Abbreviations: APS, adenosine 5′-phosphosulfate; bp, base pair(s); DTT, dithiothreitol; ELISA, enzyme-linked immunosorbant assay; HRP, horseraddish peroxidase; IPTG, isopropyl-β-D-thiogalacto-pyranoside; PAP, 3′-phosphoadenosine 5′-phosphate; PAPS, 3′-phosphoadenosine 5′-phosphosulfate; PCR, polymerase chain reaction.

2. Materials and methods

Recombinant APS-kinase was expressed from a cDNA of *Arabidopsis thaliana* L. Heynh. The identification by oligonucleotide probes of the APS-kinase cDNA and its characterisation is described in a separate publication [11]. The cDNA library was cloned in λZapII and amplified

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once [12]. In order to express the plant gene in *E. coli* (TG1 and JM81A), 161 nt of the 5'-end of cDNA were deleted by PCR using two oligonucleotides as primers: 5'-ATGGATGGATCTCAAACTCTG-3' and the reverse 3'-primer: 5'-GTATCTA↓GATCTATGTTATGCTTGAAG-3'. A mutation was introduced in the reverse primer to give an additional *Bgl*II restriction site (underlined) for unidirectional insertion. The PCR product was re-sequenced and compared with the template to eliminate errors before cloning in pBTac1 (Boehringer-Mannheim). The vector was linearized by *Eco*RI/*Bam*HI restriction which allows unidirectional ligation using the *Bgl*II/*Bam*HI hybrid at the 3' end. The overhang generated at the blunt 5'-end was filled in using Klenow fragment. In the truncated polypeptide, Met^{41} becomes the new N-terminus. Plasmids carrying the processed gene were designated pAKSS. The second construct was used for expression of a fusion protein. It was made from the same PCR fragment but using pMAL c2 (New England BioLabs Inc.) as vector. As for the pBTac constructs, insertion was unidirectional when *Xmn*I and *Bam*HI are used for the vector and *Bgl*II for the PCR fragment. The fusion protein was expressed in TB1 and isolated by affinity chromatography as outlined by the manufacturer. Factor X_a (Boehringer-Mannheim) was used to cleave the fusion protein. As a final step, APS-kinase was purified by polyacrylamide gel-electrophoresis. Standard methods used for cloning [13], DNA sequencing [14] and details of induction [11], isolation of the enzyme APS-kinase [15], HPLC measurement of APS-kinase activity [16], were as described. Polyclonal antibodies against APS-kinase were prepared as outlined by Catty [17] using homogeneous recombinant protein purified from the MAL-fusion protein by gel-electrophoresis.

3. Results and discussion

Transformation of *Escherichia coli* JM81A (*cys* C92), an APS-kinase deficient mutant, with pAKSS plasmids harbouring a truncated plant cDNA restored the capability to grow on inorganic sulfate. Prototrophic growth of this mutant indicates that the plant DNA is expressed in the bacterium and that the gene product functions as an APS-kinase. The cDNA that was cloned in pKASS has previously been identified as the plant APS-kinase DNA [11]. In order to express a functionally active gene product from the higher plant cDNA in *E. coli*, 342 bp of the 5'-terminus were deleted by PCR. This part of the DNA presumably encodes a plastidic transit peptide of 40 amino acid residues [18] which is not expected to be necessary for function. The DNA thus modified in vitro encodes a polypeptide of MW 25 761. All prototrophic transformants investigated contained the plasmid pAKSS (data not shown). In cell extracts from these bacteria analysed by SDS PAGE a new polypeptide was found which corresponded to a molecular weight of 26 kDA (data not shown) and migrating ahead of β-lactamase when the cell extracts were treated with dithiothreitol. For the only plant APS-kinase that was purified previously, a molecular weight of 44 kDa was reported [19]. We now assume that under the given conditions a homo-dimer was observed, held together by disulfide groups. It seems to be extremely stable in the presence of monothiols, so that DTT is required to monomerize the dimer. In its high content of cysteines (8 per monomer) the peptide distantly resembles the yeast enzyme which was reported to have four cysteinyl residues per monomer, two of the cysteines flanking the ATP-binding loop as in *Arabidopsis thaliana* [20]. In the plant enzyme, the additional cysteines may have a physiological function as this APS-kinase activity is controlled by thioredoxin [21].

Eight colonies from transformed JM81A harbouring the truncated plant cDNA were investigated for APS-kinase activity. In vitro rates were obtained that ranged from 10.5 to 174 nmol PAPS · mg protein^{-1} · min^{-1} (Table 1). The activity of the recombinant plant enzyme is already detectable in crude extracts; its activity is two orders of magnitude higher than the activity reported for crude extracts from the green alga *Chlamydomonas reinhardii* [10].

Considering the identity of APS-sulfotransferase, the important finding is that the same colonies harbouring pAKSS contained APS-sulfotransferase activity. The rates ranged from 0.09 to 0.240 nmol SO_3^{2-} · mg protein^{-1} · min^{-1} (Table 1). In the absence of ATP but provided with a reducing thiol like DTT, these transformed bacteria now formed sulfite from APS. This activity is not encountered in any of the wild-type or mutant strains of *E. coli* but in strains harbouring the cloned plant DNA. It represents ≈0.23% of the APS-kinase activity when assayed under conditions published for APS-sulfotransferase (i.e. 0.6 M sulfate, 30 mM thiols [22,23]). It is noteworthy, that activities reported in the literature for APS-sulfotransferases from spinach leaves [22] or *Euglena gracilis* [4] were very similar, ranging from 0.62 nmol to 0.014 nmol · mg protein^{-1} · min^{-1}.

The question of whether 'native' APS-sulfotransferase activity extracted from the intact plant is different from this cloned APS-kinase type of enzyme was investigated by Western immunoblotting. APS-sulfotransferase activity from *Arabidopsis* leaves was prepared as described for the activity from spinach [22]. In addition, as Li and Schiff [4] reported that thiols inactivated the otherwise tetrameric APS-sulfotransferase by monomerization, a separate extract was prepared omitting thiols. Both extracts were chromatographed on a HPLC gel-filtration column under conditions that supported separation under non-denaturing conditions. The resultant fractions were assayed for APS-sulfotransferase (Fig. 1A) and for APS-kinase. APS-sulfotransferase activity was only detectable in fractions that were collected from leaf extract prepared without thiols. APS-kinase activity, however, is only detectable in the presence of thiols. This is confirmed in the immunobinding assay (Fig. 1B). Fractions of the gel-filtration from both extracts were allowed to bind to nitrocellulose. Protein thus immobilized on the membrane was reacted with purified antibodies raised against recombinant APS-kinase. The resultant immunoprecipitate was then visualized using an HRP-conjugated second antibody. In leaf extract that was isolated in the presence of dithiothreitol (+DTT), the antibodies detected the low molecular weight form of the APS-kinase (≈28 kDa) almost exclusively. A very faint

Table 1
Enzyme activity as expressed in *Escherichia coli* JM81A (*cys*C) complemented with plasmids harbouring the APS-kinase cDNA from *Arabidopsis thaliana* L.

JM 81A colony complemented	APS-sulfotransferase		APS-kinase
2.1	0.17	(0.2)	85.1
2.2	0.13	(0.21)	62.3
2.3	0.11	(0.26)	42.7
2.4	0.24	(0.14)	174.0
2.5	0.12	(0.24)	51.5
2.6	0.09	(0.86)	10.5
2.7	0.1	(0.36)	26.6
2.8	0.19	(0.19)	100.1
Control	0		0

nmol PAPS or sulfite formed/mg protein · min. APS-kinase was measured by detection of PAPS employing an HPLC method [16]; APS-sulfotransferase was assayed as the amount of acid volatile SO_2 trapped in trioctylamine [11]; control, untreated host strain JM81A (*cys*C92) lacking APS-kinase. In parenthesis, sulfite formation in % of APS-kinase activity (mean 0.23 ± 0.011% for $n = 7$, excluding clone 2.6).

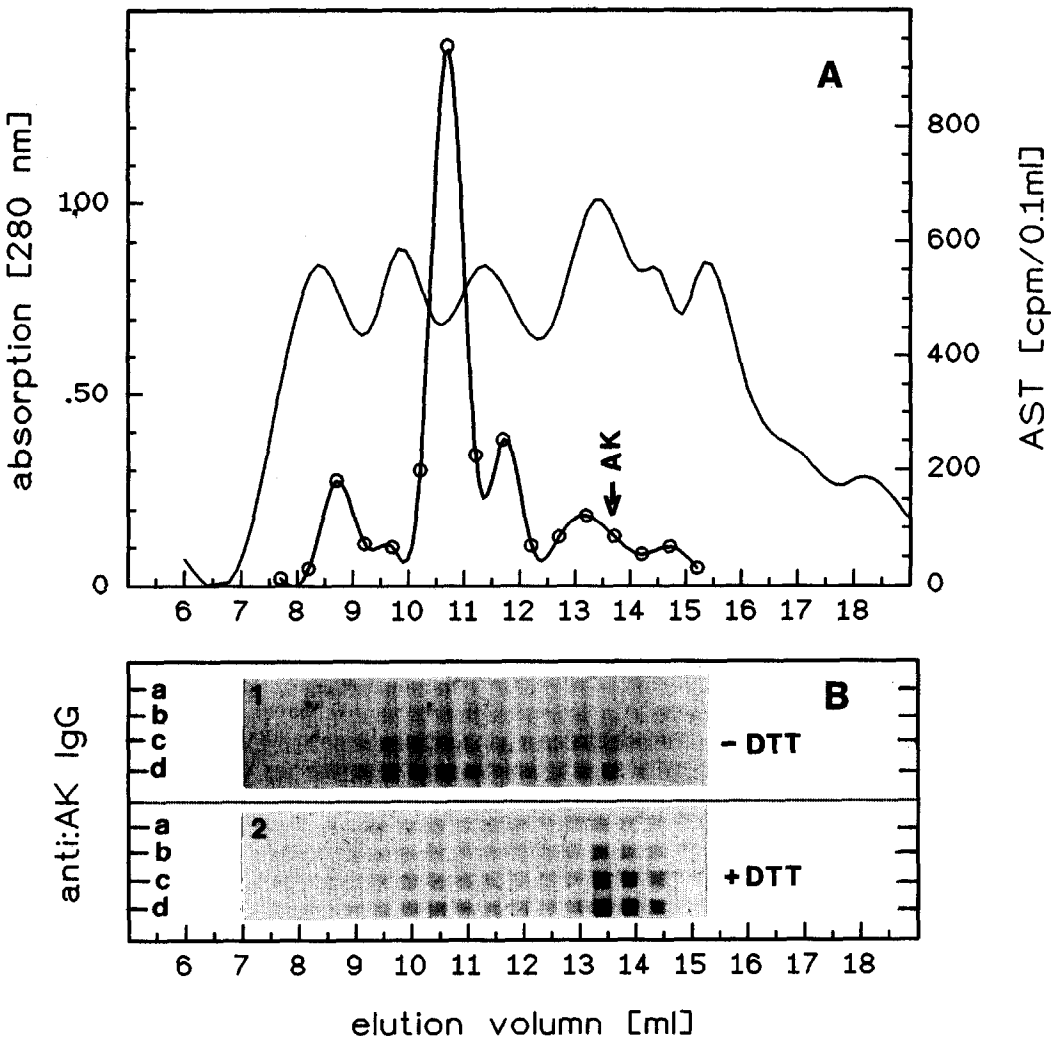

Fig. 1. Immunodetection of recombinant APS-kinase and cross-reactivity of anti-APS-kinase immunoglobulins with APS-sulfotransferase from *A. thaliana* leaf extract. (A) Protein from *Arabidopsis thaliana* leaves was separated under non-denaturing conditions by HPLC gel-filtration [24] and assayed for APS-sulfotransferase activity (○, AST, assayed as in [23]); elution volume of 10.8 ml corresponded to a molecular weight of 108 kDa; elution volume of monomeric APS-kinase (AK) 13.9 ml to 28 kDa; APS-sulfotransferase activity, $[^{35}S]SO_2$ formed from APS per 0.1 ml fraction in 15 min. (B) For Western immuno cross-blotting the leaf protein was immobilized on nitrocellulose (vertical channels, 0.2 ml of column fractions from HPLC separation) and allowed to react with purified anti-APS-kinase rabbit immunoglobulins (horizontal channels, dilution (d) 1:100, (c) 1:200, (b) 1:400, and (a) 1:800). Cross-blot 1, no thiols; cross-blot 2; leaf extract with DTT (20 mM) included. Immunoprecipitates were stained with HRP-conjugated goat anti-rabbit IgG.

precipitate of immunoreactive protein was detectable in the high molecular weight fractions but there was no enzyme activity. In leaf extract lacking thiol (−DTT), the high molecular weight form (≈ 108 kDa) prevails – under these conditions, only very small amounts of APS-kinase are detectable using the ELISA technique, or by measuring the enzyme activity (data not shown). The transition from high to low molecular weight form seems to be caused by the thiol. It may be coincidental, but APS-sulfotransferase (102 kDa) from *Euglena* [4] also disintegrates into a small subunit (≈25 kDa) upon exposure to DTT. As the antibodies are mono-specific for the plant APS-kinase, detecting the low molecular weight monomer and the higher molecular weight (tetramer?) alike, both forms must contain identical epitopes. Yet, this finding also indicates that the protein catalyzing the APS-sulfotransferase activity is identical to the gene product that is encoded by the plant APS-kinase cDNA.

The question of why plant APS-kinases have sulfotransferase activity while kinases from *E. coli* or *Saccharomyces cerevisiae* do not, is difficult to answer. All sequences of APS-kinases published until today showh highly conserved primary structures: the ATP-binding site is completely conserved as is, with one exception, a serine in the putative reaction center. From work with the APS-kinase of *E. coli* [25] it was proposed that this serine residue catalyzes (or assists in) the transfer of a phosphate group from ATP to APS by forming a phosphoserine as an intermediate. Such a mechanism would imply that APS enters the same binding niche as ATP in order to accept the phosphate group from phosphoserine. Binding of APS before ATP then may be inhibitory because ATP cannot enter. This effect may be related to the powerful substrate inhibition which is one of the most prominent properties of the enzyme besides its high affinity for APS. Hence, in the absence of ATP, one could hypothesize that APS gets trapped in the catalytic

center. In the plant APS-kinase, however, the catalytically important Ser_{182} is flanked by two cysteines (i.e. Cys_{176} and Cys_{191}). Provided that these cysteines interact with thiols in the assay mixture and with APS in the catalytic center, one could assume that the substrate is removed by a reductive cleavage in which the cysteines serve as a dithiol–disulfide redox couple. Depending on the type of exogeneous thiol used, this intramolecular reduction could either lead to enzyme-bound (with a monothiol such as glutathione) or to free sulfite (with dithiols like DTT and DTE). Whatever the mechanism, we doubt that sulfotransferase activity of the APS-kinase plays a significant role in the biosynthesis of cysteine. Rather, we propose that for cysteine biosynthesis, plants reduce PAPS to free anionic sulfite in a sequence of steps that involve APS-kinase and thioredoxin-dependent PAPS reductase as in any other prototrophic organism.

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