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Adenosine 5′-Phosphosulfate Sulfotransferase and Adenosine 5′-Phosphosulfate Reductase Are Identical Enzymes*

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Adenosine 5′-phosphosulfate (APS) sulfotransferase and APS reductase have been described as key enzymes of assimilatory sulfate reduction of plants catalyzing the reduction of APS to bound and free sulfite, respectively. APS sulfotransferase was purified to homogeneity from *Lemna minor* and compared with APS reductase previously obtained by functional complementation of a mutant strain of *Escherichia coli* with an *Arabidopsis thaliana* cDNA library. APS sulfotransferase was a homodimer with a monomer M_r of 43,000. Its amino acid sequence was 73% identical with APS reductase. APS sulfotransferase purified from *Lemna* as well as the recombinant enzyme were yellow proteins, indicating the presence of a cofactor. Like recombinant APS reductase, recombinant APS sulfotransferase used APS (K_m = 6.5 μM) and not adenosine 3′-phosphate 5′-phosphosulfate as sulfonyl donor. The V_{max} of recombinant *Lemna* APS sulfotransferase (40 μmol min^{-1} mg protein^{-1}) was about 10 times higher than the previously published V_{max} of APS reductase. The product of APS sulfotransferase from APS and GSH was almost exclusively SO_3^{2-}. Bound sulfite in the form of *S*-sulfoglutathione was only appreciably formed when oxidized glutathione was added to the incubation mixture. Because SO_3^{2-} was the first reaction product of APS sulfotransferase, this enzyme should be renamed APS reductase.

Higher plants and many microorganisms growing with sulfate as sulfur source reduce it to the level of sulfide for the synthesis of cysteine, methionine, coenzymes, and iron-sulfur clusters of enzymes (1–5). The reaction sequence from sulfate to sulfide is called assimilatory sulfate reduction as opposed to dissimilatory sulfate reduction, which occurs in certain anaerobic organisms such as *Desulfovibrio* and *Desulfotomaculum*, where sulfate functions as an electron acceptor during oxidation of organic substrates and where reduced forms of sulfur are excreted into the surroundings (3).

The first step of assimilatory sulfate reduction is an activation of sulfate catalyzed by ATP sulfurylase (EC 2.7.7.4). The adenosine 5′-phosphosulfate (APS)[1] is the substrate for APS kinase (EC 2.7.1.2.5), which forms adenosine 3′-phosphate 5′-phosphosulfate (PAPS) in a second activation step (1–5). The subsequent reduction sequence starting from PAPS is well established in bacteria (4) and fungi (6), where a PAPS reductase (EC 1.8.99) reacts first with reduced thioredoxin then with PAPS to form SO_3^{2-}, oxidized thioredoxin, and adenosine 3′-phosphate 5′-phosphate (PAP) (4, 7, 8). The SO_3^{2-} is reduced to sulfide by sulfite reductase (EC 1.8.7.1). Sulfide is finally incorporated into O-acetyl-L-serine via O-acetyl-L-serine thiollyase (EC 4.2.99.8), thus forming cysteine (1–4). All these enzymes were detected in plants (1–5, 7–11), indicating that the identical reaction sequence is operative. In two early reports it was demonstrated, however, that plants and algae use APS rather than PAPS as sulfonyl donor for the first reduction step (12, 13). APS-dependent enzymes were partially purified from spinach (14) and isolated to homogeneity from the marine macroalga *Porphyra yezoensis* Ueda (15) and from spinach leaves (16). The subunit M_r was estimated to be 43,000. The enzymes exclusively used APS as substrate and were inactive with PAPS (14–16). *In vitro* the products of the enzyme with APS and GSH as reductant were SO_3^{2-} and $GSSO_3^-$ (17–19). The consistent finding of $GSSO_3^-$ together with SO_3^{2-} when GSH was used as reductant led to the idea that GSH (19) or a small protein molecule ($CarS^-$) (18) functioned as a cofactor or carrier in a sulfotransferase reaction by accepting the sulfur group from APS to form $GSSO_3^-$ or $CarSSO_3^-$, and the enzyme was named APS sulfotransferase, accordingly. $GSSO_3^-$ and also $CarSSO_3^-$ can react nonenzymatically with thiols like GSH to form SO_3^{2-} (3, 19). It was proposed, however, that the physiological reaction pathway functioned with thiosulfonate reductase reducing the bound sulfite of $CarSSO_3^-$ to bound sulfide $CarSS^-$ (20). Thiosulfonate reductase was not characterized in detail but showed characteristics of sulfite reductase (3). Recently three isoforms of APS reducing enzymes were cloned by complementing *Escherichia coli* mutant deficient in PAPS reductase activity with an *Arabidopsis thaliana* cDNA library independently by two groups (21, 22). The cDNAs coding for these enzymes have strong homology with fungal, yeast, and bacterial PAPS reductases (21, 22), but the enzymes used APS rather than PAPS as substrate. They were named APS reductases by both groups, assuming a reaction mechanism identical to PAPS reductases in which only SO_3^{2-} was formed (21–24). Compared with PAPS reductases, APS reductase cDNAs con-

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AJ249831.

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[1] The abbreviations used are: APS, adenosine 5′-phosphosulfate; DTE, dithioerythritol; GSH, glutathione; GSSG, oxidized glutathione; $GSSO_3^-$, S-sulfoglutathione; PAPS, adenosine 3′-phosphate 5′-phosphosulfate; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; RACE, rapid amplification of cDNA ends; Bq, becquerel; RT, reverse transcription; bp, base pair(s).

tain an N-terminal extension coding for a chloroplast targeting peptide and a C-terminal extension with homology to thioredoxin (21, 22). Like APS sulfotransferases, APS reductases used DTE or GSH as a reductant (21, 25).

Here we describe the purification to homogeneity of APS sulfotransferase from *Lemna minor* and present evidence demonstrating (i) that APS sulfotransferase and APS reductase are identical enzymes, (ii) that they form SO_3^{2-} from APS with GSH as reductant, and (iii) that $GSSO_3^{2-}$ is only formed subsequently from SO_3^{2-} and GSSG.

EXPERIMENTAL PROCEDURES

Chemicals—APS and PAPS were from Sigma. All other chemicals were from Fluka (Buchs, Switzerland).

Preparative Methods—$[^{35}S]$APS and $[^{35}S]$PAPS were prepared according to Li and Schiff (26) using commercially available enzyme preparations from Sigma, containing ATP sulfurylase and APS kinase activity. $[^{35}S]$PAPS was converted into $[^{35}S]$APS by treatment with 3′-nucleotidase from Sigma (26). $[^{35}S]SO_4^{2-}$ was purchased from Amersham Pharmacia Biotech. $GSSO_3^-$ was synthesized according to Waley (27).

Plant Material—*L. minor* L. was cultivated according to Neuenschwander *et al.* (28).

Measurement of APS Sulfotransferase—Routinely APS sulfotransferase was measured according to Brunold and Suter (29). The assay systems are given in the legends of the appropriate tables and figures. Extracts from *L. minor* were prepared according to Neuenschwander *et al.* (28).

Purification of APS Sulfotransferase—The entire procedure was carried out at 0–4 °C. For the preparation of the extract 40 g of plants were rinsed with deionized water and homogenized in a glass homogenizer cooled in ice water in 160 ml of 50 mM Tris-SO_4^{2-}, pH 8.5, containing 30 mM Na_2SO_3, 10 mM DTE, 5 mM 5′-AMP, 5% polyvinylpolypyrrolidone, 0.5% polyvinylpyrrolidone, and 1 protease inhibitor tablet (Roche Molecular Biochemicals) in 50 ml. The homogenate was then filtrated through one layer of cheesecloth and centrifuged at 48,000 × *g* for 15 min. Solid $(NH_4)_2SO_4$ was slowly added to the supernatant fluid to reach 30% saturation. After 1 h of additional stirring, the mixture was centrifuged at 48,000 × *g* for 15 min. The resulting pellet was redissolved in 30 ml of standard buffer (50 mM Tris-SO_4^{2-}, pH 8.5, 30 mM Na_2SO_3, 5 mM 5′-AMP, 2 mM DTE) and one protease inhibitor tablet (Roche Molecular Biochemicals) per 50 ml. After gentle stirring the mix for 1 h, the solution fluid was applied to a 5 × 44 cm Sephacryl S-300 HR column, equilibrated with standard buffer at a flow rate of 0.9 ml min^{-1}. Proteins were eluted at the same flow rate with 800 ml of standard buffer. Fractions were collected and analyzed for APS sulfotransferase activity and protein content. Fractions with enzyme activity >50% of the peak fraction were pooled. The enzyme solution was concentrated to a final volume of 6–10 ml in an ultrafiltration cell (Amicon, model 8050) using a YM30 (30-kDa cut-off) membrane. After centrifugation at 48,000 × *g* for 15 min, the supernatant was filtrated using a disposable filter holder, mesh size 0.2 μm (FP 030/3, Schleicher & Schuell, Dassel, Germany). To carry out the three following steps a fast protein liquid chromatography system (Amersham Pharmacia Biotech) was used. The filtrate was loaded on a Mono Q HR 5/5 column equilibrated with standard buffer. The elution was performed using a linear 3-ml NaCl gradient from 50 to 100 mM, followed by 4.5 ml of standard buffer containing 1 M NaCl. Fractions were collected and analyzed for APS sulfotransferase activity and protein content. The peak fractions were pooled, and $(NH_4)_2SO_4$ was added to a final concentration of 0.6 M. This solution was applied to a Phenyl Superose HR 5/5 column equilibrated with standard buffer containing 0.7 M $(NH_4)_2SO_4$. Elution was done using a linear 9.5-ml $(NH_4)_2SO_4$ gradient in standard buffer from 0.7 M $(NH_4)_2SO_4$ to 0 mM. The peak fractions were pooled and were concentrated to a final volume of 40–80 μl using a Centricon-30 (30-kDa cut-off) ultrafiltration unit (Amicon). The concentrate was applied to a Superose 12 HR 10/30 column equilibrated with standard buffer containing 67 mM $(NH_4)_2SO_4$. Elution was carried out at a flow rate of 0.7 ml min^{-1} under isocratic conditions. The fractions with APS sulfotransferase activity >50% of the peak fraction were pooled. The enzyme solution was concentrated to a final volume of <50 μl. A native gel was prepared as described by Fankhauser and Brunold (30) omitting the stacking gel and adding 10 mM DTE and 1 mM 5′-AMP to the separating gel. To 45 μl of sample, 15 μl of loading buffer (0.5 M Tris-HCl, pH 6.8, 50% glycerol, 0.08% bromphenol blue, 0.1% mercaptoethanol) was added. The sample was loaded directly onto the gel and run in 10 mM Tris-HCl, pH 8.3, 77 mM glycine, 2 mM DTE, 1 mM 5′-AMP, 0.1% mercaptoethanol at 10 mA per gel for 4 h at 4 °C. The gel was cut into 2-mm slices. Each gel slice was placed into a elution device (Biotrap, Schleicher & Schuell), and elution took place in 15 mM NH_4HCO_3, pH 8.5, and 0.1% mercaptoethanol at 100 V for 3 h at 4 °C. The eluate was analyzed for APS sulfotransferase activity. Trichloroacetic acid was added to fractions containing activity to a final concentration of 10%. Samples were kept at 4 °C for 30 min and then centrifuged at 14,000 × *g* for another 30 min. The pellet was washed with 1 of ml acetone:H_2O = 49:1 (v/v) and redissolved in 20 μl 0.1 M DTE, 0.1 M $NaHCO_3$. 10 μl of sample buffer was added. Separation by SDS-PAGE using a Mini-Protean II slab Cell apparatus (Bio-Rad) was done on a 10% acrylamide gel as described in the manufacturer's protocol. All chromatography media, standard chromatography columns, and fast protein liquid chromatography columns were from Amersham Pharmacia Biotech.

DNA Analysis—DNA extraction and Southern hybridization were carried out according to standard procedures (31).

Isolation of Total RNA and Northern Blotting—The standard procedures of Sambrook *et al.* (31) were followed.

Amino Acid Sequencing—Gas phase microsequencing of APS sulfotransferase was done according to Söhndel *et al.* (32) after SDS-PAGE of the purified enzyme.

Isolation of APS Sulfotransferase cDNA—The central region of APS sulfotransferase was obtained from *L. minor* RNA by RT-PCR using the sequence from domains conserved between *A. thaliana* APS reductase and bacterial and yeast PAPS reductases (see Fig. 2*C*; *underlined sequences I–IV*) and the thioredoxin motif to synthesize oligonucleotide primers. The amplified fragment of 750 bp was completely sequenced on both strands. Using this sequence information, gene-specific primers (SP) for the 5′- and 3′-RACE were synthesized.

For the 5′-RACE, first strand cDNA was synthesized from 0.5 μg of poly(A) RNA using a gene-specific primer SP1 (5′-ACTGGTCCTTGCGCTGGCCG-3′), Moloney murine leukemia virus reverse transcriptase and the deoxynucleotide mixture. The mRNA-cDNA hybrid was then purified from unincorporated primers and nucleotides by the GlassMax DNA Isolation Matrix System (Life Technologies, Inc.). For adapter, 10 pmol of the oligo d(T) anchor primer (AP, 5′-GACCACGCGTATCGATGTCGACT$_{16}$-3′) and anchor reverse primer (APrev, 5′-GTCGACATCGATACGCGTGGTC-3′) were allowed to anneal. Then a blunt end ligation of the adapter and the RNA-cDNA hybrid was carried out at 15 °C overnight using 400 units T4 ligase (BioLabs).

The following PCR amplification was performed with the APrev and the nested SP2 primer (5′-GTGATCCAGGCTCTGAGGCC-3′). The PCR products were then separated on an agarose gel. For the second PCR amplification, the APrev and the third nested SP3 primer (5′-TCTGAGAGCTCTTCTAAGGGGC-3′) were used. The major band (approximately 640 bp) on the agarose gel was used as template. The PCR product was separated on an agarose gel and isolated with the GlassMax DNA Isolation Matrix System. The DNA was cloned into the pCR 2.1 vector using the Original TA Cloning Kit (Invitrogen) following the manufacturer's instructions.

For the 3′-RACE, first strand cDNA was synthesized from 0.5 μg of poly(A) RNA using the oligo d(T) anchor primer, avian murine leukemia virus reverse transcriptase, and the deoxynucleotide mixture. The first PCR amplification was then directly performed without a further purification step using the AP and the gene-specific primer SP4 (5′-ACCAGGACAGCACGAGAGGG-3′). The PCR product was separated by agarose gel electrophoresis.

For the second PCR amplification the band on the agarose gel was used as template with the nested SP5 primer (5′-AAGGTGGTGGTGGGAGGACG-3′) and the AP. After separation of the PCR products on an agarose gel the major band (approximately 560 bp) was purified using the GlassMax DNA Isolation Matrix System. The DNA was cloned into the pCR 2.1 vector using the Original TA Cloning Kit (Invitrogen) following the manufacturer's instructions.

Phylogenetic Analysis—Sequences related to PAPS and APS reducing enzymes were retrieved from GenBank™ with the BLAST software. The phylogenetic analysis was performed with the Treecon program (33). The phylogenetic tree was constructed by the neighbor-joining method (34) using the Dayhoff matrix.

Protein Overexpression and Purification—APS sulfotransferase from *L. minor* lacking the presequence encoding the transit peptide was cloned into pET14b expression vector, which ensured an addition of a His$_6$ tag at the N terminus of the expressed protein by RT-PCR amplification. For protein expression, the construct was transformed into *E. coli* BL21 (DE3). The recombinant proteins were purified from 400-ml overnight bacterial cultures on Ni^{2+} columns according to the manu-

TABLE I

Purification of APS sulfotransferase from L. minor L

The crude extract was prepared from *L. minor* containing increased APS sulfotransferase activity because of the cultivation with 10 mM NH_4Cl. 5′-AMP was included in all buffers used. The assay system contained, in a total volume of 500 µl, 100 mM Tris-HCl, pH 9.0, 800 mM $MgSO_4$, 4 mM DTE, 75 µM [^{35}S]APS (20–25 Bq nmol^{-1}), 1–10 µl extract, 200 mM Na_2SO_3. Incubation was for 30 min at 37 °C.

Purification step	Total protein	Total activity	Specific activity	Yield	Purification
	mg	*µmol min^{-1}*	*µmol min^{-1} mg protein^{-1}*	*%*	*fold*
1. Crude extract	583	11.95	0.0205	100	1
2. $(NH_4)_2SO_4$ (0–40%)	143	6.47	0.0453	54	2.2
3. Sephacryl S-300	56	4.87	0.087	41	4.2
4. Mono Q	3.8	4.16	1.095	39	53.4
5. Phenyl Superose	0.25	1.01	4.020	8.4	196.1
6. Superose 12	0.047	0.4	8.486	3.3	414.0
7. Native PAGE	0.004	0.06	15.000	0.5	731.7

facturer's instructions (Novagen).

Analysis of APS Sulfotransferase Products with APS and GSH as Substrates—The reaction mixture contained, in a final volume of 0.5 ml, 20 mM Tris-HCl, pH 9, 0.5 mM EDTA, 75 µM [^{35}S]APS, 5 mM GSH, 0, 0.5, or 1 mM GSSG, and recombinant APS sulfotransferase. This reaction mixture was optimal for product analysis but gave enzyme activities that were only approximately 10% of the maximum rates. Incubation was at 37 °C under argon. After incubation, 15 µl of 50 mM HCl and 10 µl 3.6% (w/v) formaldehyde were added. An aliquot of 0.1 ml was injected into a high performance liquid chromatography system (Kontron, Zürich, Switzerland) equipped with a 4 × 11 mm guard (column number 72 14 52 40, Marcherey Nagel) and a 4 × 250 mm anion II column (number 72 00 94, Marcherey Nagel), equilibrated with 2 mM potassium phtalate, pH 5.8. The columns were eluted using equilibration buffer from 0–18 min, followed by a linear NaCl gradient from 0 to 2 M from 18–28 min at a flow rate of 1.5 ml min^{-1}. Fractions of 0.5 ml were collected, 100 µl of 1 M Na_2SO_3 was added to each fraction, and acid volatile radioactivity was determined according to Brunold and Suter (29). Individual compounds eluted from the column were identified on the bases of their retention time, $A_{280\ nm}$, radioactivity, and capacity to liberate radioactivity upon acidification ([^{35}S]SO_3^{2-}, $GS^{35}SO_3^-$).

RESULTS

Purification of APS Sulfotransferase—Table I presents a typical purification of APS sulfotransferase from *L. minor* cultivated under aseptic condition. The activity of the enzyme was increased in the plants by addition of 10 mM NH_4Cl 24 h before harvest (35). The activity in the crude extracts varied between 20 and 25 nmol min^{-1} mg protein^{-1}. The enzyme was unstable throughout the purification procedure and during storage even though 5 mM 5′-AMP because substrate analogue for APS was added to the extraction buffer and included in all buffers used subsequently. Omission of 5′-AMP resulted in a rapid loss of enzyme activity (Fig. 1). On the gel used for native PAGE (Table I, step 7), APS sulfotransferase was visible as a yellow band, indicating a cofactor bound to the protein. From the 15 µmol min^{-1} mg protein^{-1} (Table I), it can be calculated that APS sulfotransferase contributed 1.3‰ to the total proteins in the crude extracts.

Characterization of APS Sulfotransferase—Fig. 2*A* shows an SDS-PAGE separation of three APS sulfotransferase preparations from native PAGE (Table I, step 7) differing in enzyme activity. Two bands with M_r values of approximately 41,000 and 43,000 were visible in all three lines. As expected, the fraction containing the highest APS sulfotransferase activity (Fig. 2*A*, *lane 2*) was also the one most intensely stained. The yellow color, which was visible after native PAGE, was no longer detectable after SDS-PAGE, indicating that the putative cofactor had been lost. N-terminal microsequencing of the two bands revealed an overlap of 5 amino acids (Fig. 2*B*) between the two proteins, indicating that the band with the lower M_r was a breakdown product of native APS sulfotransferase. Indeed, addition of protease inhibitors during extraction resulted in a single band after SDS-PAGE, corresponding to a M_r of 43,000 (data not shown).

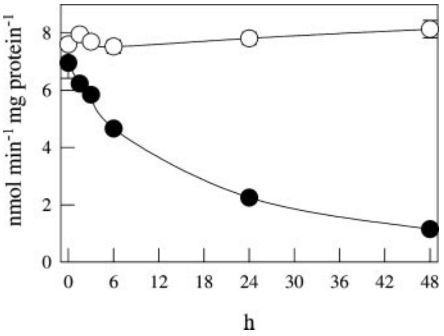
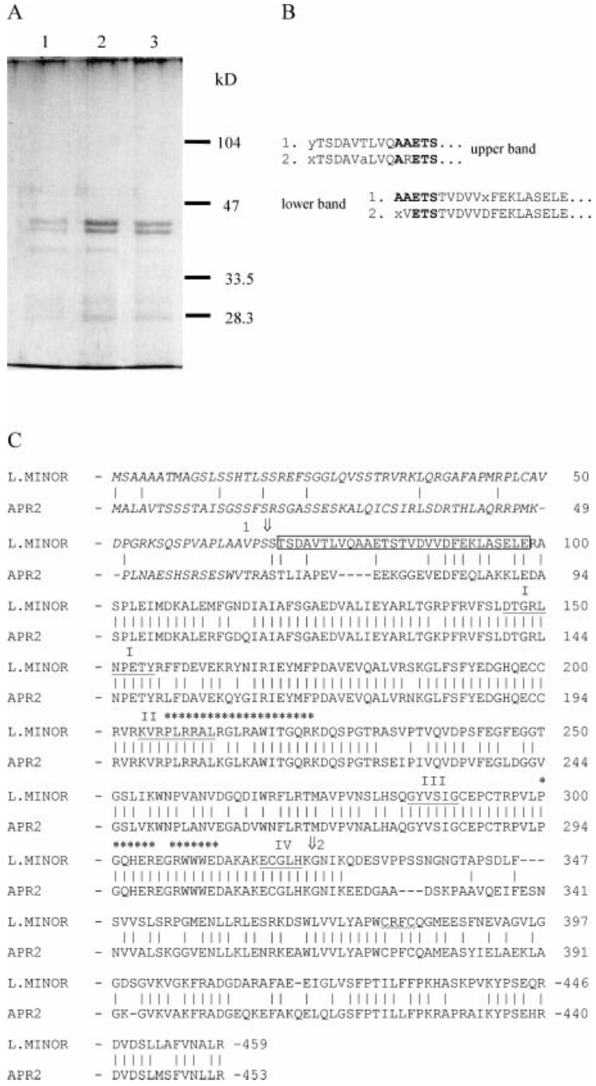


FIG. 1. **APS sulfotransferase activity in crude extracts during storage at 0–4 °C with (○) and without (●) 5 mM 5′-AMP.** The assay system described in the legend to Table I was used. The extracts added contained 26–30 µg of protein.

The complete *Lemna* APS sulfotransferase cDNA of 1377 base pairs contained a single open reading frame coding for a protein of 459 amino acids (Fig. 2*C*). The deduced amino acid sequence contained a stretch of 30 residues from Ser-69 to Glu-98 that were identical with the sequence determined from the purified APS sulfotransferase by N-terminal sequencing. This fact confirmed that: (i) this cDNA encoded the APS sulfotransferase and (ii) the encoded protein possessed a presequence coding for a chloroplast transit peptide. The mature APS sulfotransferase is thus composed of 391 amino acids with a calculated molecular weight of 43,638, which is in good agreement with the results of the SDS-PAGE. The deduced amino acid sequence of the mature APS sulfotransferase is 73% identical to the APS reductase from *A. thaliana*. Similarly to APS reductase, APS sulfotransferase consists of two major domains homologous to PAPS reductase and thioredoxin. The N-terminal part of both enzymes up to the beginning of the thioredoxin-like extension was 82% identical.

In addition to the two cysteines of the thioredoxin motive (Fig. 2*C*) and a cysteine in Lys^{317}-Glu-Cys-Gly-Leu-His, a putative motive for interaction with S-nucleotides also present in PAPS reductases, both APS reductase and APS sulfotransferase contain two more cysteine pairs. These findings taken together indicate that the APS sulfotransferase protein isolated from *L. minor* is highly similar to APS reductase from *A. thaliana*. Estimation of the M_r by gel filtration using Superose 12 resulted in a M_r of 91,300 ± 7,640, indicating that native APS sulfotransferase is composed of two identical subunits (data not shown).

Southern blot analysis (Fig. 3) performed with a 750-bp central fragment of APS sulfotransferase cDNA indicated that probably more than one gene for this enzyme is present in *L. minor*. However, even though seven different RT-PCR products were sequenced, no polymorphisms were observed, indicating



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1. yTSDAVTLVQAAETS...  upper band
2. xTSDAVaLVQARETS...

lower band   1. AAETSTVDVVxFEKLASELE...
             2. xVETSTVDVVDFEKLASELE...
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L.MINOR  - MSAAAATMAGSLSSHTLSSREFSGGLQVSSTRVRKLQRGAFAPMRPLCAV   50
           |  |        |    |    |            |        |
APR2     - MALAVTSSSTAISGSSFSRSGASSESKALQICSIRLSDRTHLAQRRPMK-   49
                          1 ⇓
L.MINOR  - DPGRKSQSPVAPLAAVPSSTSDAVTLVQAAETSTVDVVDFEKLASELERA  100
            |                ||  |       |     | ||| ||  || |
APR2     - -PLNAESHSRSESWVTRASTLIAPEV----EEKGGEVEDFEQLAKKLEDA   94
                                                          I
L.MINOR  - SPLEIMDKALEMFGNDIAIAFSGAEDVALIEYARLTGRPFRVFSLDTGRL  150
           ||||||||||| ||  |||||||||||||||||||||| |||||||||||
APR2     - SPLEIMDKALERFGDQIAIAFSGAEDVALIEYARLTGKPFRVFSLDTGRL  144
            I
L.MINOR  - NPETYRFFDEVEKRYNIRIEYMFPDAVEVQALVRSKGLFSFYEDGHQECC  200
           |||||| || ||| | |||||||||||||||||| |||||||||||||||
APR2     - NPETYRLFDAVEKQYGIRIEYMFPDAVEVQALVRNKGLFSFYEDGHQECC  194
              II **********************
L.MINOR  - RVRKVRPLRRALRGLRAWITGQRKDQSPGTRASVPTVQVDPSFEGFEGGT  250
           |||||||||||| || |||||||||||||||   | ||||| |||  ||
APR2     - RVRKVRPLRRALKGLKAWITGQRKDQSPGTRSEIPIVQVDPVFEGLDGGV  244
                                               III          *
L.MINOR  - GSLIKWNPVANVDGQDIWRFLRTMAVPVNSLHSQGYVSIGCEPCTRPVLP  300
           ||| |||| ||| | | | ||||| ||||| || ||||||||||||||||
APR2     - GSLVKWNPLANVEGADVWNFLRTMDVPVNALHAQGYVSIGCEPCTRPVLP  294
           ****** *******     IV ⇓2
L.MINOR  - GQHEREGRWWWEDAKAKECGLHKGNIKQDESVPPSSNGNGTAPSDLF---  347
           |||||||||||||||||||||||||||              |    |
APR2     - GQHEREGRWWWEDAKAKECGLHKGNIKEEDGAA---DSKPAAVQEIFESN  341

L.MINOR  - SVVSLSRPGMENLLRLESRKDSWLVVLYAPWCRFCQGMEESFNEVAGVLG  397
            || ||  | |||| || ||  |||||||||| ||| || |  | |  |
APR2     - NVVALSKGGVENLLKLENRKEAWLVVLYAPWCPFCQAMEASYIELAEKLA  391

L.MINOR  - GDSGVKVGKFRADGDARAFAE-EIGLVSFPTILFFPKHASKPVKYPSEQR -446
           |  |||| ||||||    ||  |  | |||||| ||| |    ||||| |
APR2     - GK-GVKVAKFRADGEQKEFAKQELQLGSFPTILLFPKRAPRAIKYPSEHR -440

L.MINOR  - DVDSLLAFVNALR -459
           |||||  ||| ||
APR2     - DVDSLMSFVNLLR -453
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FIG. 2. **SDS-PAGE separation of three APS sulfotransferase preparations obtained by elution of gel slices from native PAGE. (*A*, purification step 7 in Table I), N-terminal amino acid sequences of the proteins separated by SDS-PAGE (*B*), and amino acid sequence deduced from APS sulfotransferase cDNA of *L. minor* compared with APS reductase of *A. thaliana* (*C*).** Relative enzyme activity of the three APS sulfotransferase preparations loaded on SDS-PAGE were 8:40:21 for lines 1, 2, and 3, respectively (*A*). The amino acid sequences of the two bands from SDS-PAGE indicating an overlap are presented in *bold letters* (*B*). In the amino acid sequence of *L. minor* APS sulfotransferase (*C*), the sequence obtained by combining the sequences of *B* are *boxed*. Conserved amino acid regions homologous to PAPS reductase are *underlined* in the *L. minor* APS sulfotransferase sequence and numbered *I–IV*. These regions were used for preparing oligonucleotides for RT-PCR reactions with *L. minor* RNA. *Arrow 1* indicates the cleavage site of the transit peptide identified on the bases of the N-terminal sequence of isolated *L. minor* APS sulfotransferase protein; *arrow 2* indicates the beginning of the thioredoxin-like portion. The sequences of the *Lemna* APS sulfotransferase indicated by *asterisks* were used for preparing oligonucleotides for the 3′- and 5′-RACE. The *waved line* indicates the thioredoxin motif.

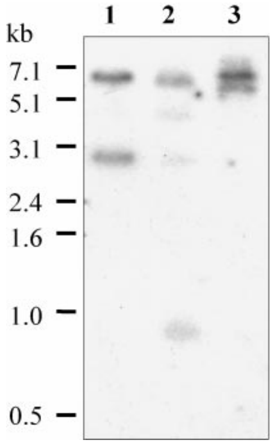

FIG. 3. **Southern analysis of *L. minor* genomic DNA.** The DNA from *L. minor* was cut with *Hin*dIII (*lane 1*), *Eco*RI (*lane 2*), and *Nde*I (*lane 3*) and hybridized with the 750-bp central fragment of APS sulfotransferase cDNA.

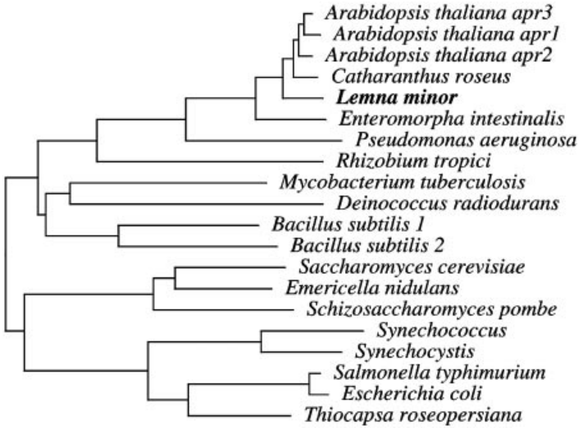

FIG. 4. **Phylogenetic tree of APS and PAPS reducing enzymes.** Microbial PAPS reductase amino acid sequences were retrieved from GenBank™ using the blast software and aligned with the N-terminal domain of plant APS reductases. This alignment was used to construct a neighbor-joining tree by the Treecon program (37).

that this is the predominant APS sulfotransferase isoform expressed under the cultivation conditions applied.

The amino acid sequence of APS sulfotransferase was used to search the GenBank™ data base for related sequences. Only the N-terminal PAPS reductase-like domains of plant APS reductases were used for further analysis. A neighbor-joining tree was constructed from these sequences (Fig. 4). The close relationship of *L. minor* APS sulfotransferase with APS reductases of *A. thaliana*, *Catharathus roseus*, and the marine algae *Enteromorpha intestinalis* is clearly evident. On the other hand, the group comprising well studied PAPS reductases from *E. coli* and *Salmonella typhimurium* as well as the enzymes from yeast and cyanobacteria are only distantly related to plant APS reductases. Surprisingly, the plant APS reductases are closely related to the *CysH* of *Pseudomonas aeruginosa* and *Rhizobium tropici*.

Properties of APS Sulfotransferase—Table II shows that recombinant APS sulfotransferase was dependent on APS as sulfonyl donor that cannot be replaced by PAPS. This is in contrast to crude extracts where enzyme activities obtained with PAPS were comparable with those with APS (data not shown). DTE can be replaced by GSH as a reductant in the assay for APS sulfotransferase.

The effect of APS, GSH, and 5′-AMP on the activity of recombinant APS sulfotransferase from *L. minor* is presented in Fig. 5. Both, APS and GSH were inhibitory at high concentrations (Fig. 5, *A* and *B*). The $K_{m\ \mathrm{APS}}$ was not significantly affected by increasing the GSH concentration from a physiological level of 10 mM to 40 mM (Fig. 5*A*). At 40 μM APS the $K_{m\ \mathrm{GSH}}$ was 16.8 ± 5.3 mM (Fig. 5*B*). The effect of 5′-AMP on $K_{m\ \mathrm{APS}}$ but not $V_{\max}$ (Fig. 5*C*) indicates a competitive inhibition, which is consistent with its chemical structure being similar to APS. A $K_{i\ 5'\text{-AMP}}$ of 0.34 ± 0.06 and 0.24 ± 0.04 mM was calculated from measurements with 10 (Fig. 5*C*) and 40 (data not shown) mM GSH, respectively. If we assume that APS sulfotransferase

TABLE II

Requirements of recombinant APS sulfotransferase from L. minor

The enzyme assay described in the legend of Table I was used. [^{35}S]APS had a specific radioactivity of 73.8 Bq nmol^{-1}, [^{35}S]PAPS of 37.7 Bq nmol^{-1}. Recombinant APS sulfotransferase was added in amounts of 24–59 ng. Four independent measurements ± S.D. are presented.

Assay system	Activity
	μmol min^{-1} mg protein^{-1}
Complete	12.4 ± 2.7
−APS + 75 μM PAPS	0.014 ± 0.008
−DTE + 10 mM GSH	6.5 ± 3.1
−DTE	0.07 ± 0.028

operates as a dimer and use a $V_{\max}$ of 39.2 μmol min^{-1} mg protein^{-1} as enzyme activity, a turnover number of 56 s^{-1} can be calculated.

In Vivo Effect of NH_4^+ on APS Sulfotransferase Activity and mRNA—The APS sulfotransferase activity in crude extracts of *Lemna* plants growing with NO_3^- as sole the nitrogen source was 100–150 pmol·min^{-1} mg fresh weight^{-1} (Fig. 6*A*). When NH_4^+ was added to the culture medium as an additional nitrogen source 24 h before using *Lemna* as material for APS sulfotransferase isolation, the enzyme activity was increased by 50–100% (Fig. 6*A*). This increase of enzymatic activity correlated well with the preceding increase of APS sulfotransferase mRNA (Fig. 6*B*). It has been shown before that *L. minor* cultivated with NH_4^+ for 24 h contained approximately 15% more protein than cultures with NO_3^- as the sole nitrogen source (35). The increase of APS sulfotransferase activity may be necessary, therefore, for providing the sulfur amino acids needed for the increased protein synthesis.

Products of APS Sulfotransferase—The product(s) of APS reductase were not determined but was presumed to be SO_3^{2-} (23–25). In contrast, $GSSO_3^-$ (17) or $GSSO_3^-$ together with SO_3^{2-} (18) were consistently described as *in vitro* reaction products of APS sulfotransferase with GSH as reductant. Here, APS sulfotransferase product formation was analyzed under an atmosphere of argon, preventing oxidation reactions, and in the presence of EDTA for complexing heavy metals. Without these precautions, SO_3^{2-} was rapidly oxidized to SO_4^{2-} (data not shown). Fig. 7*A* shows that recombinant APS sulfotransferase from *L. minor* almost exclusively produced SO_3^{2-} from APS and GSH. $GSSO_3^-$ formation was only detected in appreciable amounts when 1 mM GSSG was added to the incubation mixture (Fig. 7*B*). This result clearly indicated that the first product of the enzyme reaction was SO_3^{2-} and led to the hypothesis that $GSSO_3^-$ was formed from GSSG and SO_3^{2-}. Consistent with this hypothesis, $GSSO_3^-$ in the assay system increased with increasing GSSG concentrations (data not shown) and also with time, both in absolute (Fig. 8*A*) and relative (Fig. 8*B*) amounts, whereas relative SO_3^{2-} levels decreased (Fig. 8*B*), even though SO_3^{2-} increased linearly in absolute amounts (Fig. 8*A*). The very small amount of SO_3^{2-} present at time 0 (Fig. 8*A*) was formed between addition of [^{35}S]APS to the assay system and addition of HCl and formaldehyde for stopping the reaction. No $GSSO_3^-$ could be detected at this time point. The hypothesis was further tested in an experiment in which 1 mM unlabeled $GSSO_3^-$ was added to the assay system before incubation. If radioactive $GSSO_3^-$ was the first product of the enzyme reaction, it would be trapped by unlabeled $GSSO_3^-$. Consistent with the results of Figs. 7 and 8, however, radioactivity in the $GSSO_3^-$ pool was not significantly increased in this experiment (data not shown), corroborating the hypothesis that SO_3^{2-} was indeed the first enzyme product.

This led to the question of whether $GSSO_3^-$ was formed chemically or enzymatically. To answer this question, 5 or 100

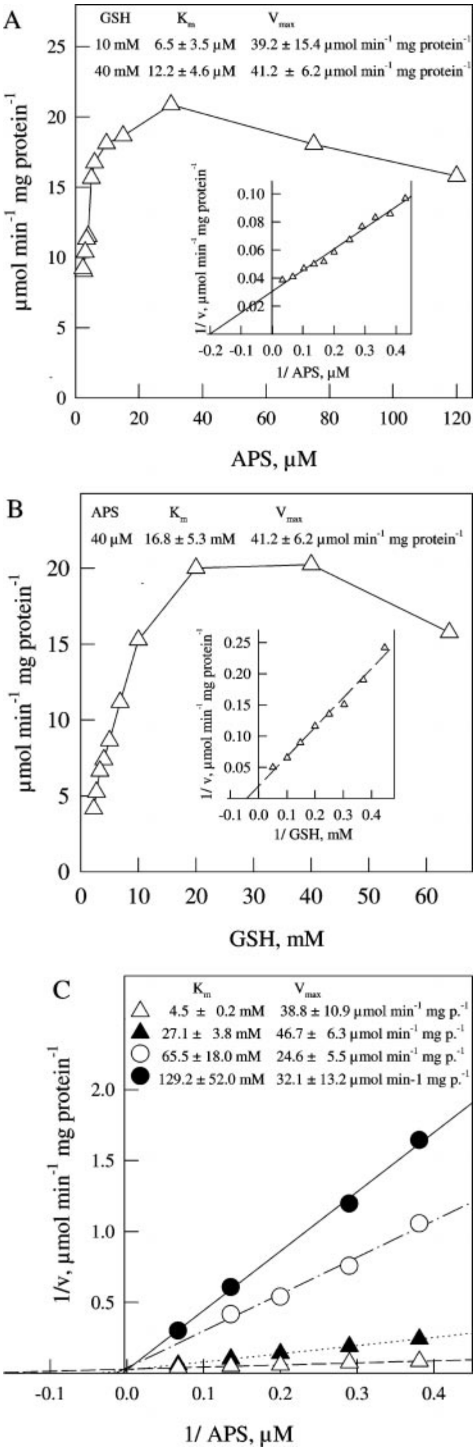

FIG. 5. **Effect of APS (*A*), GSH (*B*), and 5′-AMP (*C*) on the activity of recombinant APS sulfotransferase from *L. minor*.** The assay systems corresponded to those given in the legend of Table I. For *A*, APS was varied between 2325 and 120 μM, and GSH was 10 mM; for *B*, GSH was varied between 2.25 and 64 mM, and APS was 40 μM; and for *C*, GSH was 10 mM, and APS varied between 2.625 and 15 μM, and 0 (△), 0.5 (▲), 2.5 (○), or 5 (●) mM 5′-AMP were added. The APS sulfotransferase preparations had a specific activity of 20–25 μmol min^{-1} mg protein^{-1}. Incubation was for 15 min at 37 °C, [^{35}S]APS used had a specific radioactivity of 73.8 Bq nmol^{-1}. Results from a typical experiment presented as Michaelis-Menten (*A* and *B*) and Linewearer-Burk (*A*—*C*, *insets*) plots and from four independent experiments presented as the mean values ± S.D. are shown.

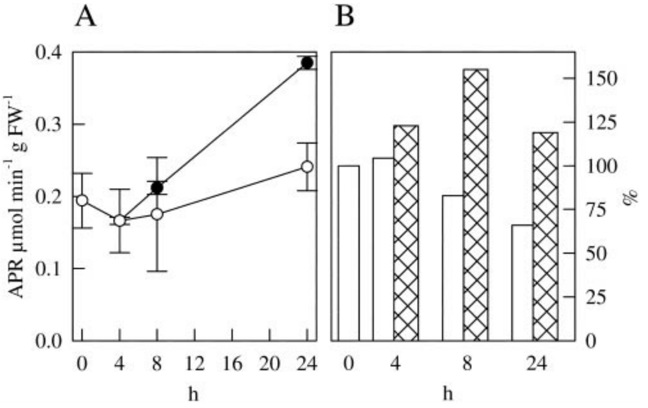

FIG. 6. **Activity (*A*) and mRNA (*B*) of APS sulfotransferase from *L. minor* after addition of 10 mM NH_4Cl.** Crude extracts were used for measuring APS sulfotransferase activity in the assay system presented in the legend of Table I. Values from six to eight measurements ± S.D. are presented for both control (○) and NH_4 Cl (●) cultures. The 750-bp central fragment of APS sulfotransferase cDNA was used for hybridization. The mRNA values are means of two experiments. Relative mRNA values were obtained using a PhosphoImager and taking the value at the start of the experiment as 100%.

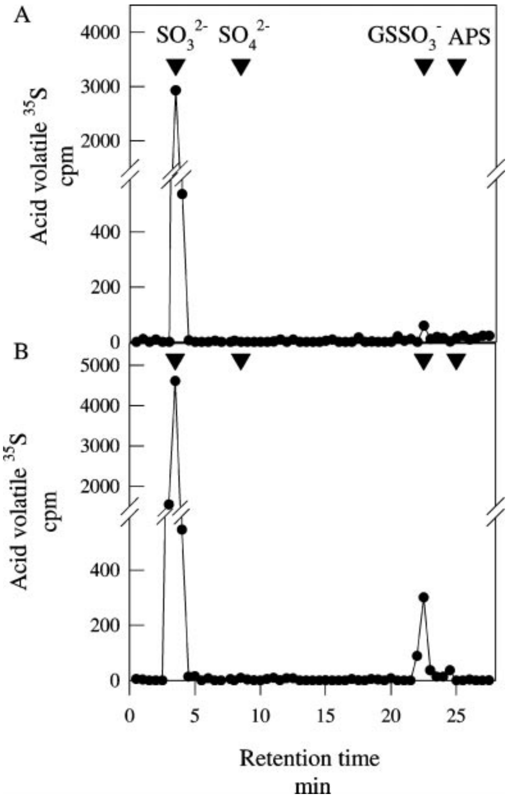

FIG. 7. **Products of recombinant APS sulfotransferase from *L. minor* using APS as substrate and GSH as reductant.** The assay was done as described under "Experimental Procedures." $[^{35}S]$APS had a specific activity of 33.9 Bq nmol^{-1} for *A* and of 82.4 for *B*. The activity of APS sulfotransferase was measured under optimal conditions, and 338 ng of recombinant enzyme with an activity of 26 μmol^{-1} min^{-1} mg protein^{-1} and 676 ng with an activity of 17 μmol^{-1} min^{-1} mg protein^{-1} were used in *A* and *B*, respectively. Incubation was for 10 min. The peaks representing acid volatile radioactivity correspond to 3.3 μmol^{-1} min^{-1} mg protein^{-1} SO_3^{2-} in *A* and to 1.05 μmol min^{-1} mg protein^{-1} SO_3^{2-} and 0.45 nmol in each assay $GSSO_3^-$ in *B*.

μM radioactive SO_3^{2-} were incubated with 1 mM GSSG. In this system, radioactive $GSSO_3^-$ was formed at the same rate, independent of the presence of APS sulfotransferase, indicating that SO_3^{2-} reacted with $GSSO_3^-$ chemically rather than enzymatically (data not shown).

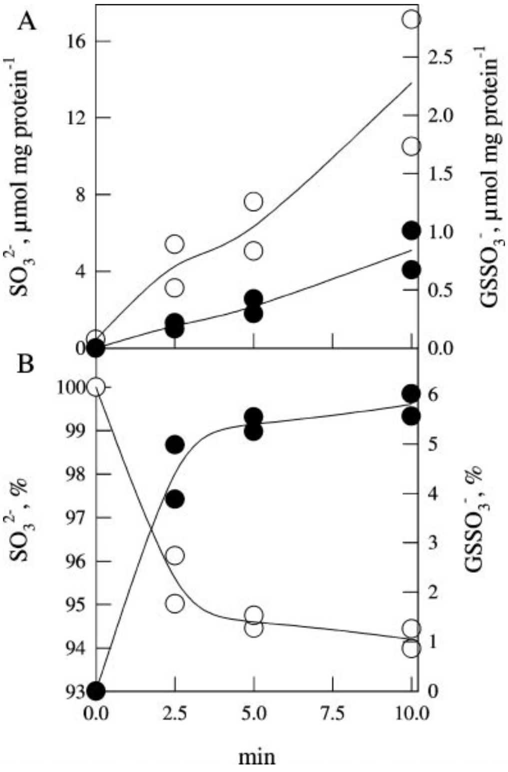

FIG. 8. **Time course of SO_3^- (○) and $GSSO_3^-$ (●) formation in the incubation mixture of APS sulfotransferase.** 1 mM GSSG was added to the incubation mixture given in Fig. 7. Results from two independent experiments are presented at each time point. The recombinant APS sulfotransferase activity measured under optimal conditions varied between 16 and 19 μmol min^{-1} mg protein^{-1}. 338 and 676 ng of APS sulfotransferase were added to the incubation mixture.

DISCUSSION

The evidence presented here shows that APS sulfotransferase characterized after enzyme purification from *L. minor* and APS reductase isolated by complementation of *E. coli* mutants with *A. thaliana* cDNA library (21, 22) are identical enzymes. The following evidence demonstrates that the cDNA isolated from *L. minor* RNA codes for the protein that had been purified to homogeneity beforehand: (i) the N-terminal amino acids obtained by protein sequencing and deduced from cDNA were identical, (ii) the M_r deduced from the cDNA was identical to the M_r of the purified protein, (iii) radioactive probes synthesized with the isolated cDNA detected a mRNA of the expected size, and (iv) the isolated cDNA functioned as template for the synthesis of a recombinant enzyme with APS reducing activity. Because it was demonstrated here that the first product of this enzyme was SO_3^{2-} and not $GSSO_3^-$, it seems appropriate to replace the name APS sulfotransferase by APS reductase. Therefore, only the term APS reductase will be used in the following discussion. The sequence of APS reductase encoding a chloroplast transit peptide indicates a plastid localization of the enzyme (21, 22). Indeed, APS reductase was shown to be localized exclusively or almost exclusively in the chloroplasts of spinach leaves (36) and in the proplastids of pea roots (37). Even though APS reductases have a PAPS reductase portion, they are exclusively or almost exclusively active with APS. A PAPS reductase has been isolated from spinach leaves (9), which leads to the question of the physiological relevance of APS-dependent enzymes in sulfate assimilation. Several lines of evidence indicate that in higher plants, APS rather than PAPS-dependent enzymes are predominantly contributing to sulfate assimilation: (i) even though 100 different colonies were analyzed,[2] after complementation of PAPS reductase-deficient *E. coli* mutants (21, 22) using a *A. thaliana* cDNA library only APS-dependent enzymes could be isolated; this finding indicates that PAPS reductase mRNA, should it exist in *A. thaliana*, is present only in low abundance; (ii) consistent with this idea, the activity of partially purified PAPS reductase from spinach (9) amounts to only 0.94 nmol min^{-1} mg protein^{-1} compared with 10–25 nmol min^{-1} mg protein^{-1} for APS reductase in crude extracts of *L. minor*; (iii) in agreement with this big difference in enzyme activity, isotope dilution experiments with crude plant extracts indicate that PAPS is not appreciably used directly as a sulfonyl donor but is degraded to APS by a 3′-nucleotidase before being reduced (38, 39). These experiments also explain why PAPS was used as a sulfonyl donor by crude *L. minor* extracts at comparable rates as APS; (iv) when more reduced sulfur was needed for establishing increased protein levels in the presence of NH_4^+ (38), APS reductase activity and mRNA increased accordingly (Fig. 6). This result corroborates previous findings demonstrating that this enzyme activity is increased under conditions of increased need for reduced sulfur compounds (1, 2); (v) both APS reductase activity and mRNA were induced under conditions of sulfur deficiency (22, 40, 41), which represents additional physiological evidence for the participation of this enzyme in assimilatory sulfate reduction of plants.

The concentration of GSH in the chloroplast may be up to 4.5 mM (43), which is lower than the $K_{m\ \mathrm{GSH}}$ (16.7 mM; Fig. 5*B*) but still sufficient for high *in vivo* APS reductase activity. The APS concentration in plant chloroplasts has not been determined yet. According to Siegel (3), an accumulation of APS to a level of approximately 10^{-5} M could be expected at equilibrium, if ATP is present at 10^{-4} M, whereas SO_4^{2-} and P_i are each present at 10^{-3} M. The APS reductase isolated from *L. minor* had a $K_{m\ \mathrm{APS}}$ of 6.5 μM at 10 mM GSH (Fig. 5), indicating that the APS calculated to be present *in vivo* in the chloroplasts may be sufficient for obtaining even more than half-maximum rates.

Previously $GSSO_3^-$ (17, 18) was reported as product of APS reductase, whereas the results presented here showed that $GSSO_3^-$ was formed from SO_3^{2-} and GSSG. The previously published results can therefore be best explained by assuming that under the conditions used, GSSG was formed in the reaction mixture at appreciable rates. In the studies presented here, conditions including elimination of O_2 and chelating of heavy metals minimized chemical formation of GSSG from GSH. GSSG resulting from GSH used as reductant for the APS reductase reaction gave a final concentration of approximately 20 μM (Fig. 7). This concentration was too low for producing appreciable amounts of $GSSO_3^-$. *In vivo* SO_3^{2-} formed by APS reductase can be used directly by sulfite reductase (3, 11). The low K_m for SO_3^{2-} of this enzyme together with its high activity (11) will prevent the accumulation of high SO_3^{2-} concentrations *in vivo*. Because also GSSG is at a low level in plant material under normal conditions (42), $GSSO_3^{2-}$ is not expected to accumulate to high concentrations. Should it be formed, however, it can either be reduced to GSH and SO_3^{2-} by glutathione reductase (43) or react nonenzymatically with GSH to form GSSG and SO_3^{2-} (3).

The yellow color of the purified APS reductase brings to mind the molecular structure of dissimilatory APS reductases which contain FAD and iron-sulfur clusters (3). Indeed, a corresponding analysis of recombinant APS reductase from *A. thaliana* showed that this enzyme contained iron and had an absorption spectrum typical of enzymes with iron-sulfur clusters (44). Very recently a recombinant APS reductase from *C. roseus* was reported (25) to have no color and a V_{max} more than 10 times lower than V_{max} of the recombinant enzyme from *L. minor*. This finding can be explained by assuming that no iron-sulfur cluster was incorporated into the recombinant *C. roseus* APS reductase, resulting in decreased enzyme activity. The loss of activity during purification of APS sulfotransferase may also be due to loss of a putative iron-sulfur cluster as has been reported for enzymes containing iron-sulfur clusters (45).

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